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CLINICAL AND EXPERIMENTAL

A HORMONALLY CONTROLLED SERUM FACTOR WHICH STIMULATES SULFATE INCORPORATION BY CARTILAGE IN VITRO

WILLIAM D. SALMON, JR., M.D.,* AND WILLIAM H. DAUGHADAY, M.D.
ST. LOUIS, MO.

CLINICAL and experimental evidence has established the importance of the pituitary growth hormone in promoting skeletal growth. Marked impairment of osteogenesis and chondrogenesis occurs after hypophysectomy in the rat, and these processes are stimulated by the administration of growth hormone.[1] Despite continuous research for many years, the mechanism of these actions of growth hormone remains obscure.

To gain information concerning hormonal stimulation of skeletal growth, we have measured the incorporation of S^{35}-sulfate by cartilage in vitro. Both in vivo[2, 3] and in vitro,[4] cartilage has been shown to incorporate S^{35}-sulfate into chondroitin sulfate. Twenty-four hours after the administration of S^{35}-sulfate to intact rats, the decline of radioactivity in cartilage, as determined by quantitative autoradiography, has been found to occur at a rate similar to the decline in radioactivity of the ester sulfate of isolated chondroitin sulfate.[5] It has been shown also that practically no sulfate sulfur is incorporated into cystine[6, 7] or methionine.[7] Only the latter of these sulfur-containing amino

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acids, and only in small amounts, has been demonstrated in collagen,[8, 9] the other major component of the intercellular substance of cartilage. Therefore, one is justified in interpreting stimulation of sulfate uptake by cartilage as indicative of increased incorporation into chondroitin sulfate.

Earlier work by Ellis, Huble, and Simpson,[10] by Denko and Bergenstal,[11] and in our own laboratory,[12] had shown that uptake of sulfate by cartilage in vivo in rats was greatly reduced by hypophysectomy and restored by growth hormone. In this paper, we wish to report the stimulation in vitro of sulfate incorporation into cartilage from hypophysectomized rats by a factor in the serum of normal rats and the appearance of this factor in the plasma after treatment of hypophysectomized rats with growth hormone.

METHODS

Preparation of Animals.—Male rats of the Sprague-Dawley strain were used exclusively. Hypophysectomy* was performed at 21 ± 2 days of age. The rats were maintained under standard conditions[12] until experiments were conducted 12 to 20 days after operation. The animals were decapitated and blood was collected with or without powdered heparin.†

Conditions of Incubation.—In most experiments, costal cartilage was employed; in a few, nasal or xiphoid cartilage was used. After removing soft tissues and bone, the specimens were placed in individual glass vials (17 × 60 mm.) containing 0.5 ml. of Krebs' phosphosaline buffer,[13] plasma, serum, or serum diluted with buffer. To each vial was added 0.2 ml. of Krebs's phosphosaline buffer containing 1.4 mg. of glucose, 35 units of penicillin, 35 mcg. of streptomycin, and 0.23 to 0.33 μc of carrier-free S^{35}-sulfate.‡ The vials were stoppered and incubated at 37° C. for 24 hours, unless otherwise indicated.

Determination of Radioactivity.—After incubation, the cartilages were placed in individual gauze bags with metal markers, immersed in boiling water for 3 minutes to stop enzymatic action, and washed for 3 hours in running tap water to remove inorganic S^{35}-sulfate. Any adherent soft tissue was removed, and, after blotting, the cartilages were weighed on a torsion balance to the nearest tenth of a milligram. Each specimen was placed in a tube containing 2 ml. of 0.2 M sodium sulfate and 2 ml. of concentrated hydrochloric acid, and the tube and contents were heated for 3 hours in a steam bath. The sulfate was isolated as barium sulfate and radioactivity determined as previously described.[12] The results were expressed as counts per minute per milligram of wet cartilage, measured under the conditions described, or converted to sulfate uptake in micrograms per 100 mg. of wet cartilage by calculation from the specific activity of the incubation media.

Other Analytical Methods.—For determinations of the serum inorganic sulfate, proteins were precipitated with 3 volumes of 12 per cent trichloracetic acid; the sulfate was precipitated with benzidine.[14] The benzidine sulfate was washed with alcohol and dissolved in 0.2 N hydrochloric acid. The benzidine was diazotized and determined colorimetrically.[15]

The serum glucose concentration was determined by the procedure of Nelson,[16] serum proteins by the method of Weichselbaum.[17]

Statistical Methods.—Analysis of the results was carried out by the "t" test, the analysis of variance and the "F" test, and, for calculation of regression, the method of least squares.[18]

*Hypophysectomy performed by the Hormone Assay Laboratory, Chicago, Ill.
†Heparin: Connaught Laboratories, University of Toronto, Toronto, Canada.
‡Obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

Each experiment was designed to be self-contained. The experimental design using multiple costal cartilages from the same animal in incubations under various conditions has permitted the very significant animal variation to be circumvented in the analysis of the data. The coefficient of variation for error (includes variation due to counting, differences between individual costal cartilages from the same rat) has ranged from 9.8 to 14 per cent, with the median between 11 and 12 per cent.

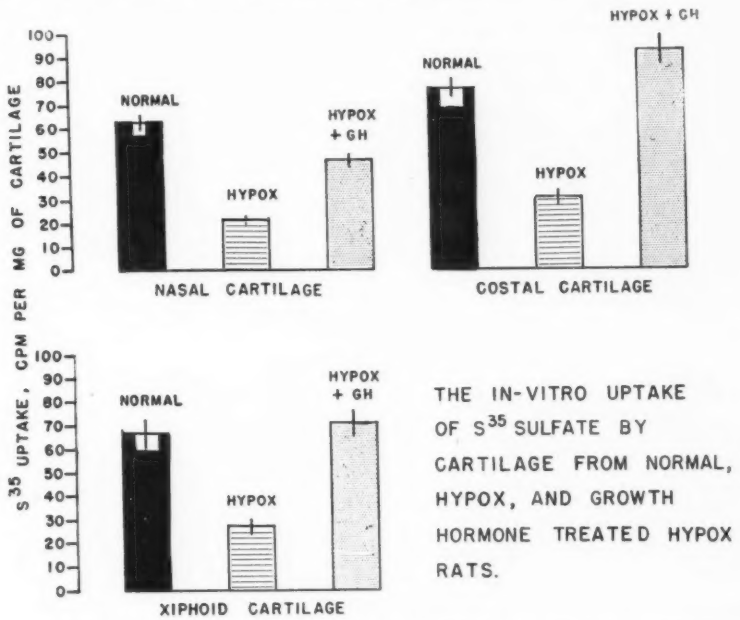
S^{35} UPTAKE, CPM PER MG OF CARTILAGE
NORMAL
HYPOX
HYPOX + GH
NASAL CARTILAGE
COSTAL CARTILAGE
XIPHOID CARTILAGE
THE IN-VITRO UPTAKE OF S^{35} SULFATE BY CARTILAGE FROM NORMAL, HYPOX, AND GROWTH HORMONE TREATED HYPOX RATS.

Fig. 1.—A comparison of the uptake of S^{35}-sulfate in vitro by cartilage from normal rats, hypophysectomized rats, and growth hormone-treated hypophysectomized rats. Each bar represents the mean for a single determination in each of 6 rats. The vertical line at the top of the bar represents ± the standard error of the mean. In the last group of rats, Armour Beef Growth Hormone J21609R, 1.0 mg. total dose per rat, was administered in 3 injections intraperitoneally 42, 24, and 16 hours before the rats were killed. In this and other figures and tables: Hypox = hypophysectomized; GH = growth hormone.

RESULTS

Nasal, xiphoid, and costal cartilages from normal rats, hypophysectomized rats, and growth hormone-treated, hypophysectomized rats were compared as to their sulfate incorporation during incubation in vitro (Fig. 1). The mean sulfate uptake by cartilage from hypophysectomized rats was less than one-half the uptake in the corresponding cartilage from normal rats. The mean sulfate uptake by cartilage from growth hormone-treated, hypophysectomized rats was similar to that of cartilage from normal rats.

The sulfate uptake of cartilage from hypophysectomized rats was then measured after incubation in different types of rat plasma (Table I). The mean sulfate uptake of nasal and costal cartilage incubated in plasma from normal rats, Experiments A and C, was greater than twice that of cartilage incubated in plasma from hypophysectomized rats ($P < .001$). The plasma from growth hormone-treated, hypophysectomized rats, Experiment B, stimulated sulfate uptake in xiphoid cartilage to greater than twice the uptake found with incubation in plasma from hypophysectomized rats ($P < .003$).

TABLE I. EFFECT OF PLASMA FROM HYPOPHYSECTOMIZED RATS, NORMAL RATS, AND GROWTH HORMONE–TREATED, HYPOPHYSECTOMIZED RATS ON UPTAKE OF SULFATE IN VITRO BY CARTILAGE FROM HYPOPHYSECTOMIZED RATS

EXPERIMENT	INCUBATION MEDIUM†	CARTILAGE	NO. OF RATS (CARTILAGE DONORS)	S^{35}-SULFATE UPTAKE IN CPM PER MG. WET CARTILAGE (MEAN ± S.E.)
A	Hypox rat plasma	Nasal	6	50.6 ± 3.0
	Normal rat plasma	Nasal	6	147.4 ± 6.5
B	Hypox rat plasma	Xiphoid	5	47.0 ± 3.8
	GH-treated,* hypox rat plasma	Xiphoid	6	120.6 ± 9.2
C	Hypox rat plasma	Costal	5	60.3 ± 4.9
	Normal rat plasma	Costal	5	144.0 ± 10.1

*Armour Beef Growth Hormone J21609R, 1.0 mg. total dose per rat in 3 injections intraperitoneally 42, 24, and 16 hours before rats were killed.

†Media: 0.5 ml. plasma plus 0.2 ml. Krebs-Ringer bicarbonate buffer (Umbreit, W. W., Burris, R. H., and Stauffer, J. F.: Manometric Techniques and Tissue Metabolism. Minneapolis, 1949, Burgess Publishing Company, p. 119), containing additives, incubated in atmosphere of 95 per cent O_2, 5 per cent CO_2 (Experiment A and B); 0.5 ml. plasma plus 0.2 ml. Krebs' phosphosaline buffer containing additives, incubated in atmosphere of room air (Experiment C).

The time of appearance of sulfation-promoting activity in the plasma of hypophysectomized rats after growth hormone treatment was investigated by incubating costal cartilage from hypophysectomized rats with the plasma of groups of rats killed at various intervals, from 2 to 24 hours, after the hormone injection (Fig. 2). There was no significant increase in sulfation-promoting activity in plasma from the rats killed 2 hours after growth hormone administration. However, plasma obtained 6 hours after growth hormone administration produced a highly significant ($P < .001$) stimulation of sulfate uptake by cartilage in vitro, and the mean sulfate uptake by cartilage incubated in plasma obtained 12 hours after treatment with growth hormone was 96 per cent greater than the control uptake by cartilage incubated in plasma from untreated hypophysectomized rats. The sulfate uptake in vitro by costal cartilage removed from the growth hormone-treated rats and incubated in a buffer medium was also measured in this experiment (Fig. 2). There was not a significant difference in sulfate uptake between cartilage from untreated hypophysectomized rats and cartilage from hypophysectomized rats killed 2 hours after the injection of growth hormone. However, the sulfate uptake in vitro by cartilage from hypophysectomized rats killed 6 hours after treatment with growth hormone was significantly ($P < .05$) greater than the sulfate uptake by cartilage from untreated control rats. The further increase in sulfate uptake by cartilage from the rats killed 12 hours after treatment was highly significant ($P < .001$). The mean sulfate uptake by cartilage from the rats killed 24 hours after treatment with growth hormone was more that 2½ times the control uptake.

When growth hormone was added in vitro in a buffer medium to costal cartilage from hypophysectomized rats (Table II, Experiments A and B), there was a slight stimulation of sulfate uptake, significant when compared

with the control only in the concentration of 50 meg. per milliliter. The augmentation at this concentration was only 18 to 19 per cent. Although the effects at 2 and 5 meg. per milliliter were not significant when compared with the control, the regression of sulfate uptake on hormone concentration, calculated from the analysis of variance, was significant ($P < .03$). Growth hormone, up to 0.14 meg. per milliliter, added to plasma from hypophysectomized rats (Table II, Experiment C), did not cause an increase in sulfate uptake by costal cartilage from hypophysectomized rats.

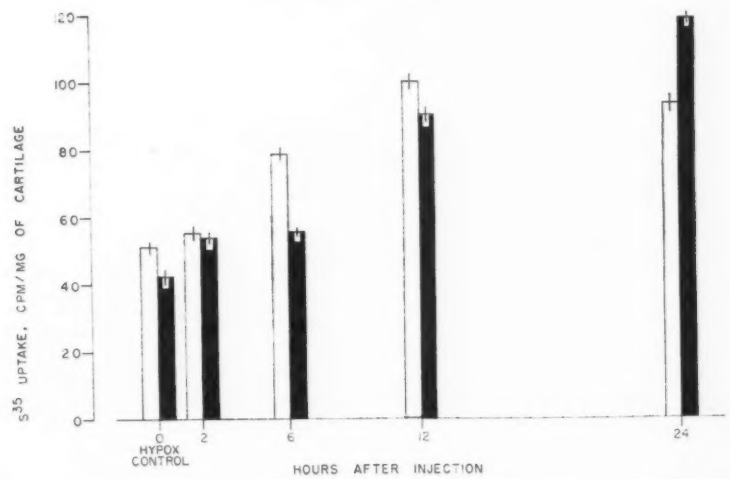

Fig. 2.—The effect of plasma from hypophysectomized rats treated with growth hormone at various intervals prior to obtaining the plasma on the uptake of S^{35}-sulfate in vitro by costal cartilage from hypophysectomized rats. The control group of hypophysectomized rats received no hormone. Each bar represents the mean for a single determination in each of 6 hypophysectomized rats serving as cartilage donors. Also shown is the uptake of S^{35}-sulfate in vitro by costal cartilage removed from the control and the various growth hormone-treated groups. Each bar represents the mean for triplicate determinations in each of 5 rats. The vertical line at the top of each bar represents ± the standard error of the mean. In this experiment Armour Beef Growth Hormone M108 was injected intraperitoneally in a dose of 500 meg. per rat. Costal cartilage from injected rats incubated in Krebs' phosphosaline buffer (solid bars); costal cartilage from hypox rats incubated with plasma from injected rats (plain bars).

Insulin, added in vitro in a buffer medium, stimulated sulfate uptake by costal cartilage from hypophysectomized rats (Table III, Experiment A). There was a highly significant ($P < .001$), positive correlation ($r = 0.72$) between sulfate uptake and insulin concentration from 0.001 to 1.0 unit per milliliter. When compared with the control, the differences at the concentrations of 0.001 and 0.01 unit per milliliter were not significant at the 5 per cent level. Significant stimulation was seen at 0.1 ($P < .002$) and 1.0 ($P < .001$) unit per milliliter, the mean increase over the control at the latter concentration being 62 per cent. Significant ($P < .003$) stimulation of sulfate uptake also resulted when insulin was incubated with costal cartilage from normal rats fasted for 24 hours prior to the experiment (Table III, Experiment B). Insulin, 0.1 unit per milliliter, had no effect on sulfate uptake by costal cartilage from growth hormone-treated, hypophysectomized rats (Table III, Experiment C).

TABLE II. EFFECT OF GROWTH HORMONE ADDED IN VITRO ON SULFATE UPTAKE OF COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS

EXPERIMENT	INCUBATION MEDIUM	COSTAL CARTILAGE SAMPLES	GROWTH HORMONE* (MCG./ML.)	S^{35}-SULFATE UPTAKE IN CPM PER MG. OF CARTILAGE (MEAN ± S.E.)
A	KPS† buffer	2 from each of 5 rats at each dose level	0	24.0 ± 1.1
			2	25.5 ± 1.4
			5	26.4 ± 1.2
			50	28.2 ± 1.3 $P < .02$
B	KPS buffer	3 from each of 6 rats at each dose level	0	19.7 ± 1.4
			50	23.5 ± 1.6 $P < .001$
C	Hypox rat plasma	1 from each of 6 rats at each dose level	0	28.8 ± 2.6
			0.0014	23.9 ± 1.0
			0.014	28.8 ± 1.8
			0.14	29.8 ± 2.5

*Armour Beef Growth Hormone D728-076 (Experiment A and B), J21609R (Experiment C).

†KPS = Krebs' phosphosaline.

TABLE III. EFFECT OF INSULIN ADDED IN VITRO IN A BUFFER MEDIUM ON SULFATE UPTAKE OF COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS, NORMAL RATS, AND GROWTH HORMONE-TREATED, HYPOPHYSECTOMIZED RATS

EXPERIMENT	COSTAL CARTILAGE DONOR RATS	NO. OF RATS	INSULIN* (UNITS/ML.)	S^{35}-SULFATE UPTAKE—MEAN ± S.E. CPM/MG. WET CARTILAGE	S^{35}-SULFATE UPTAKE—MEAN ± S.E. PER CENT INCREASE OVER CONTROL‡
A	Hypox	6	0	29.1 ± 1.4	
		6	0.001	33.9 ± 0.8	17.4 ± 3.3
		6	0.01	31.5 ± 1.7	8.8 ± 2.8
		5	0.1	37.2 ± 3.3	31.9 ± 6.4
		6	1.0	46.4 ± 2.8	61.6 ± 12.4
B	Normal, fed	6	0	88.7 ± 4.2	
		6	0.14	101.0 ± 6.9	13.7 ± 5.3
	Normal, fasted 24 hours	6	0	73.0 ± 4.4	
		6	0.14	94.6 ± 4.9	30.8 ± 6.7
C	GH-treated,† hypox	5	0	58.2 ± 4.4	
		5	0.1	58.3 ± 3.2	0.6 ± 2.5

*Insulin: Iletin, Eli Lilly Company, Indianapolis, Ind.

†Armour Beef Growth Hormone M108 0.5 mg. each intraperitoneally 24 hours before animals were killed.

‡Calculated on the basis of each rat as its own control.

In other experiments, not shown, L-thyroxine* and L-triiodothyronine,† added in vitro in a buffer medium, had no effect on sulfate incorporation by costal cartilage from hypophysectomized rats.

In further studies of the stimulation observed with addition in vitro of normal serum to costal cartilage from hypophysectomized rats, the absolute sulfate uptake per 100 mg. of wet cartilage was calculated from the specific activity of the incubation media, determined from the serum inorganic sulfate, the sulfate content of the buffer, and the added radioactivity. The mean

*L-thyroxine sodium: Travenol Laboratories, Inc., Morton Grove, Ill.

†L-triiodothyronine sodium: Research Laboratories, Smith, Kline and French, Philadelphia, Pa.

sulfate concentration, in millimoles per liter, in 10 pools of serum from hypophysectomized rats was 1.256 (range 1.100 to 1.574), in 8 pools of serum from normal rats 1.156 (range 1.096 to 1.277), and in 6 pools of serum from growth hormone-treated, hypophysectomized rats 1.264 (range 1.170 to 1.420).

Increasing the time of incubation in a buffer medium from 1 to 18 hours had no effect on the rate of sulfate uptake by costal cartilage from hypophysectomized rats. However, with incubation in serum from normal rats the rate of sulfate uptake by this cartilage increased progressively during the incubation (Fig. 3). At the end of the first hour, there was no significant

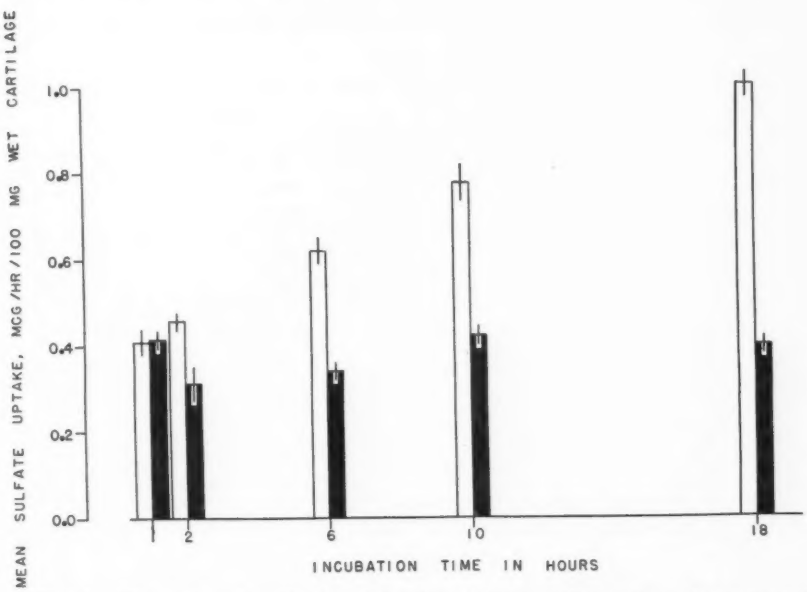

Fig. 3.—The rate of sulfate uptake by costal cartilage from hypophysectomized rats during various periods of incubation with serum from normal rats, as compared with incubation in a buffer medium. Each bar represents the mean for a single determination in each of 6 rats serving as cartilage donors. The vertical line at the top of each bar represents ± the standard error of the mean. Normal rat serum (plain bars); Krebs' phosphosaline buffer (solid bars).

difference between incubation in the 2 media, the mean sulfate uptake per 100 mg. of wet cartilage being 0.41 ± 0.03 mcg. in the serum and 0.42 ± 0.02 mcg. in the buffer. However, at 18 hours the mean sulfate uptake per hour for the entire period was 1.01 ± 0.03 mcg. per 100 mg. of wet cartilage in the serum and still only 0.40 ± 0.02 mcg. in the buffer. Estimating by difference, the mean sulfate uptake per hour between 10 and 18 hours in the serum was 1.30 mcg. per 100 mg. of wet cartilage, and the most rapid increase in uptake occurred between 2 and 10 hours.

The effect of dilution of serum from normal rats on its sulfation-promoting activity, when incubated with costal cartilage from hypophysectomized rats, was investigated (Fig. 4). Two serum pools, each from a group of 30 normal rats, were tested in separate experiments. The rats in Group A weighed 120

to 145 grams and in Group B, 95 to 115 grams. A highly significant ($P < .001$) linear relationship was found between the per cent increase over control sulfate uptake by costal cartilage from hypophysectomized rats and the logarithm of the volume of serum added to the medium. The 95 per cent confidence limits for the regression coefficient in Group A were 104.9 to 174.7, in Group B, 65.3 to 147.5. The difference between the regression coefficients was tested and found significant ($P < .05$). However, the procedure used was not identical in the 2 experiments, since the mean of single determinations in each of 6 rats serving as cartilage donors was used for each point with serum pool A and the mean of duplicate determinations in each of 6 rats for each point with serum pool B. There was considerably more variation with the

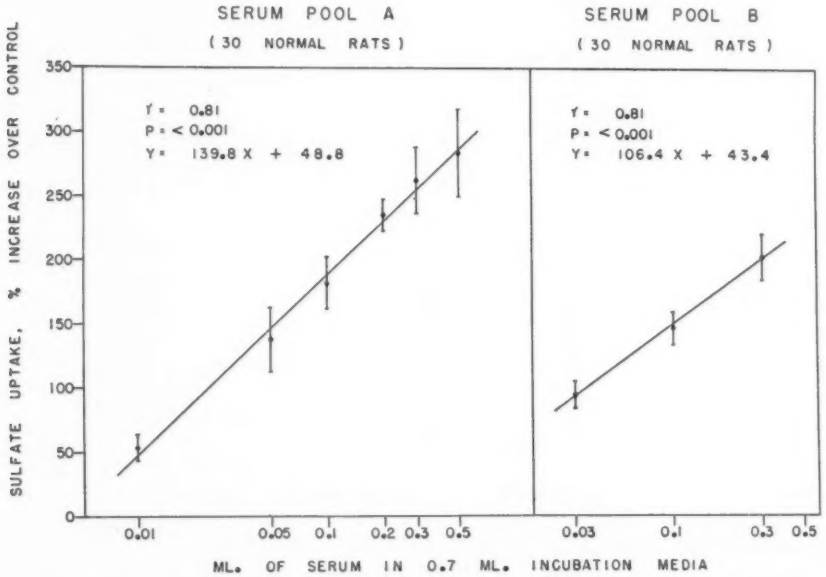

Fig. 4.—The stimulatory effect of graded amounts of serum from normal rats on the uptake of sulfate in vitro by costal cartilage from hypophysectomized rats. Each point in the experiment utilizing serum pool A represents the mean for a single determination in each of 6 rats serving as cartilage donors. Each point in the experiment utilizing serum pool B represents the mean for duplicate determinations in each of 6 rats serving as cartilage donors. The brackets indicate ± the standard error of the mean. In the regression equations, Y = the mean uptake of sulfate by cartilage, as per cent increase over the control, and X = the logarithm of 100 X the volume of serum added to the incubation medium.

former procedure, as would be expected. When the method used with the serum pool B was followed in another experiment, not shown, using serum from another group of 30 normal rats, a regression coefficient of 97.5, with 95 per cent confidence limits of 64.4 to 130.6, was found. There is no significant difference between this regression coefficient and the value of 106.4 found earlier using serum pool B.

To determine the effect of dialysis on the sulfation-promoting activity of serum, in 2 experiments (Table IV) serum from hypophysectomized rats and from normal rats was dialyzed against Krebs' phosphosaline buffer and then

incubated with costal cartilage from hypophysectomized rats. Before dialysis of the serum, the uptake of sulfate by cartilage incubated with serum from normal rats was slightly over 100 per cent greater than the uptake by cartilage incubated with serum from hypophysectomized rats. The sulfate uptake by cartilage incubated in the latter medium was, however, greater than in the buffer alone; this was observed also in other experiments. When serum dialyzed for 4 hours was used for the incubation medium (Experiment B), the sulfate uptake by cartilage was 75 per cent greater in the serum from normal rats than in the serum from hypophysectomized rats. In a similar experiment, not shown, the sulfate uptake of cartilage incubated in serum dialyzed for 4 hours was 99 per cent greater in the serum from normal rats than in the serum from hypophysectomized rats. When the incubation medium contained serum dialyzed for 24 hours, the sulfate uptake by cartilage was 79 (Experiment A) and 44 (Experiment B) per cent greater in the serum from normal rats than in the serum from hypophysectomized rats.

TABLE IV. EFFECT OF DIALYSIS ON SERUM SULFATION ACTIVITY

EXPERIMENT	INCUBATION MEDIUM	NO. OF RATS (CARTILAGE DONORS)	SULFATE UPTAKE IN MCG. PER 100 MG. OF WET CARTILAGE (MEAN ± S.E.)		
			BEFORE DIALYSIS	AFTER 4-HOUR DIALYSIS*	AFTER 24-HOUR DIALYSIS*
A	KPS buffer	7	5.1 ± 0.4		
	Hypox rat serum	7	7.3 ± 0.5		5.2 ± 0.2
	Normal rat serum	7	15.8 ± 0.8		9.3 ± 0.7
B	KPS buffer	6	7.9 ± 0.3		
	Hypox rat serum	6	9.4 ± 0.5	7.9 ± 0.3	7.9 ± 0.3
	Normal rat serum	6	19.0 ± 0.6	13.8 ± 0.5	11.4 ± 0.3

*Serum dialyzed in a cellophane bag against 100 volumes of Krebs' phosphosaline buffer, in 2 equal portions, at 4° C.

Recently Boström and co-workers[19] have described a "sulfate exchange stimulating factor," effecting an increase in sulfate incorporation in vitro by slices of costal cartilage from suckling calves. This thermostabile, dialyzable factor was found in homogenates of brain, liver, muscle, heart, and lung of rats and in serum and liver of a number of species. In further studies of the liver factor they have identified this as glutamine.[20] When incubated according to the procedure reported in the present paper, lyophilized aqueous extracts of livers from normal rats and hypophysectomized rats, prepared according to Boström's method for beef liver,[21] had no effect, in the concentrations used, on sulfate uptake by costal cartilage from hypophysectomized rats (Table V, Experiment A). Under similar conditions of incubation there was no effect on sulfate incorporation by costal cartilage from either normal or hypophysectomized rats when L-glutamine was added to the medium (Table V, Experiment B). The concentrations of glutamine used in this experiment are 1½ to 15 times the mean concentration found in blood of normal rats.[22] Furthermore, the addition of L-glutamine to an incubation medium containing

dialyzed serum from either normal or hypophysectomized rats did not increase sulfate uptake by costal cartilage from hypophysectomized rats (Table V, Experiment C).

TABLE V. EFFECT OF AN EXTRACT OF RAT LIVER AND OF GLUTAMINE ON SULFATE UPTAKE OF COSTAL CARTILAGE

EXPERIMENT	INCUBATION MEDIUM	COSTAL CARTILAGE	CARTILAGE SULFATE UPTAKE PER CENT INCREASE OVER CONTROL ± S.E.
A	KPS buffer + hypox rat liver extract 143 mg. %	2 from each of 5 hypox rats	8.0 ± 8.4
	KPS buffer ± normal rat liver extract 143 mg. %	2 from each of 5 hypox rats	1.8 ± 5.2
B	KPS buffer + glutamine* 10 mg. %	2 from each of 6 hypox rats	0.2 ± 4.9
	KPS buffer + glutamine* 100 mg. %	2 from each of 6 hypox rats	−14.0 ± 4.3
	KPS buffer + glutamine* 10 mg. %	2 from each of 6 normal rats	−1.4 ± 2.1
	KPS buffer + glutamine* 100 mg. %	2 from each of 6 normal rats	1.6 ± 3.6
C	Dialyzed hypox rat serum 0.2 ml. + KPS buffer 0.5 ml. + glutamine* 10 mg. %	1 from each of 6 hypox rats	0.9 ± 7.0
	Dialyzed normal rat serum 0.2 ml. + KPS buffer 0.5 ml. + glutamine* 10 mg. %	1 from each of 5 hypox rats	−13.5 ± 4.3

*L (+)-glutamine (natural): Nutritional Biochemicals Corporation, Cleveland, Ohio.

DISCUSSION

The effects of hypophysectomy and growth hormone administration on sulfate uptake of cartilage in intact rats[10-12] have been confirmed by an in vitro technique. Extension of these experiments has demonstrated that uptake of sulfate by cartilage from hypophysectomized rats can be stimulated in vitro by incubation of the cartilage with serum from either normal rats or hypophysectomized rats treated with growth hormone. The stimulating component of serum has been designated sulfation factor.[23] Optimum conditions for induction of this factor in the serum of hypophysectomized rats by the administration of growth hormone have not been established, and some variability in response has been observed.

The potency of the sulfation factor is indicated by the finding that addition to a buffer medium of only 0.01 ml. of serum from normal rats (1:70 dilution) resulted in over 40 per cent mean increase in sulfate uptake in vitro by costal cartilage from hypophysectomized rats. The linear relationship between the sulfate uptake by cartilage from hypophysectomized rats, expressed as per cent increase over the control, and the logarithm of the volume of serum added to the incubation medium may provide a convenient assay for the sulfation factor.

At present there is little information concerning the chemical nature of the sulfation factor. It seems reasonably certain that this factor is not identical with either growth hormone or insulin. Only minimal stimulation in vitro

of sulfate incorporation by cartilage from hypophysectomized rats was found with high concentrations of growth hormone. The stimulation observed with insulin is likewise minimal in concentrations which might be considered physiologic for the normal rat (around 0.01 unit of insulin-like activity per milliliter of serum, using the rat diaphragm assay, according to Randle[24]). It also appears reasonable to conclude that the serum sulfation factor which is active in stimulating sulfation by rat cartilage is distinct from glutamine, a substance which stimulates sulfation by slices of beef cartilage. The explanation for this species specificity of glutamine is unknown. The persistence of considerable sulfation-promoting activity after 24 hours dialysis of serum from normal rats suggests that an active component is either protein in nature or highly protein-bound. Little is known about the stability of the substance, but serum stored at –20° C. for long periods or heated to 60° C. for 15 minutes retains activity.

CONCLUSIONS

1. The incorporation in vitro of sulfate by cartilage from rats is reduced by hypophysectomy and can be restored by growth hormone administered in vivo.

2. Serum from normal rats contains a factor, termed sulfation factor, which promotes sulfate uptake in vitro by cartilage from hypophysectomized rats.

3. The sulfation factor appears to be growth hormone-induced, since it appears in the plasma of hypophysectomized rats after the administration of growth hormone.

4. Considerable sulfation-promoting activity can still be demonstrated after high dilution or extensive dialysis of serum from normal rats.

5. The sulfation-promoting effect of serum from normal rats, reported in these experiments, is not the result of its glutamine content.

The authors are indebted to Dr. Eric Reiss of the Department of Medicine for advice on statistics and to Dr. David Brown of the Department of Biological Chemistry for his assistance with analytic methods.

ADDENDUM

In recent experiments, to be published later, the sulfation-promoting activity lost on dialysis of serum from normal rats has been recovered in the dialysate. The activity of the original serum was restored by combining the dialyzed serum with the dialysate. Additional evidence indicates that at least a part of the dialyzable sulfation-promoting activity can be attributed to essential amino acids.

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CHANCE DISTRIBUTION AND THE PLACEBO "REACTOR"

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IT HAS been proposed that experimental subjects may be separated according to their proclivity for reacting to placebo medication.[1-5] The present study was undertaken to test the consistency with which placebo responses occurred from individual to individual and in the same individual from time to time.

METHODS

Data from an earlier study of agents tested for their ability to prevent ipecac-induced nausea and vomiting showed that none was more effective or more consistent in its effect than a placebo.[6] Since none of the agents showed evidence of pharmacodynamic activity, they were all regarded as placebos. The experimental group consisted of 21 volunteers who had consistently exhibited nausea upon the ingestion of ipecac alone on two separate occasions and 14 who had consistently exhibited vomiting. Each underwent 7 additional trials with ipecac preceded by oral administration of the above agents according to a double-blind systematized randomization technique. Both the subjects and the investigator were aware that an antiemetic effect was being sought. Following each of their 7 trials, a subject's failure to develop the anticipated nausea and/or vomiting was designated as a placebo response. Those who consistently displayed placebo responses or consistently failed to do so were called pure reactors or nonreactors, respectively; those who displayed placebo responses on exactly half the trials were called half-reactors; and those who had more placebo responses than nonresponses, but were not totally consistent, were termed impure reactors (or impure nonreactors, if they demonstrated more nonresponses than responses).

These data were particularly suitable for testing the concept of the placebo reactor because both a subjective response (nausea) and an objective response (vomiting) were observed. Accordingly, the distribution of complete "protection" against nausea in the 21 subjects and of complete "protection" against vomiting in the 14 subjects, as well as the distribution of partial "protection" against either nausea or vomiting, was recorded and compared to the theoretical distribution attributable to chance as derived from the binomial expansion equation. Further, the total of all placebo responses in the 35 subjects were compiled and compared again to the appropriate chance curve. Finally, the variation in the occurrence of placebo responses from time to time in the same individual was compared with that observed from person to person. Since the number of tests performed on each subject was 7, the subjects were divided into groups of 7 and each was analyzed from the standpoint of the variations described above.

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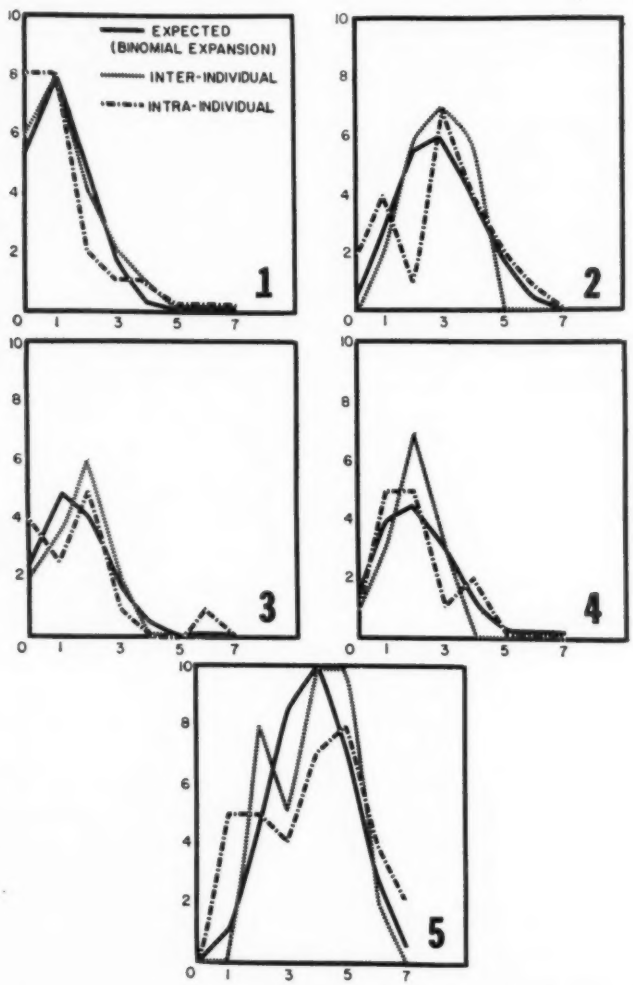
RESULTS

In Figs. 1 and 2, the interindividual and intraindividual variations in the incidence of placebo responses with respect to nausea are compared with the appropriate chance curve derived from the binomial expansion equation. Fig. 1 gives data for complete "protection" against nausea and Fig. 2 gives data for partial "protection." It will be seen that the curves for variation in



Figs. 1 to 5.—All figures represent the interindividual and the intraindividual variation in response to a placebo compared to the theoretical variation due to chance as derived from the binomial expansion equation.

The abscissa represents the number of placebo responses possible in 7 trials on different subjects (for interindividual) and in 7 trials on the same subject (for intraindividual). The ordinate indicates the number of occasions on which the placebo responses were observed among the groups in each category.

Fig. 1 represents the data for complete "protection" against nausea and Fig. 2 represents the data for partial "protection" (21 subjects, each with 7 successive placebo tests). Fig. 3 represents the data for complete "protection" against vomiting and Fig. 4 the data for partial "protection" (14 subjects, each with 7 successive placebo tests). Fig. 5 represents the combined data for both complete and partial "protection" against both nausea and vomiting (35 subjects, 7 successive placebo tests each).

placebo response, both inter- and intraindividual, did not differ from each other or from chance. Figs. 3 and 4 illustrate, in the same manner, the data on complete and partial "protection" against vomiting. Again there is no significant difference between the observed results and chance. Fig. 5 shows the combined data compared to its binomial expansion curve and again there are no significant differences. In each instance, the data were subjected to chi-square analysis by which the lack of any significant differences was established mathematically.

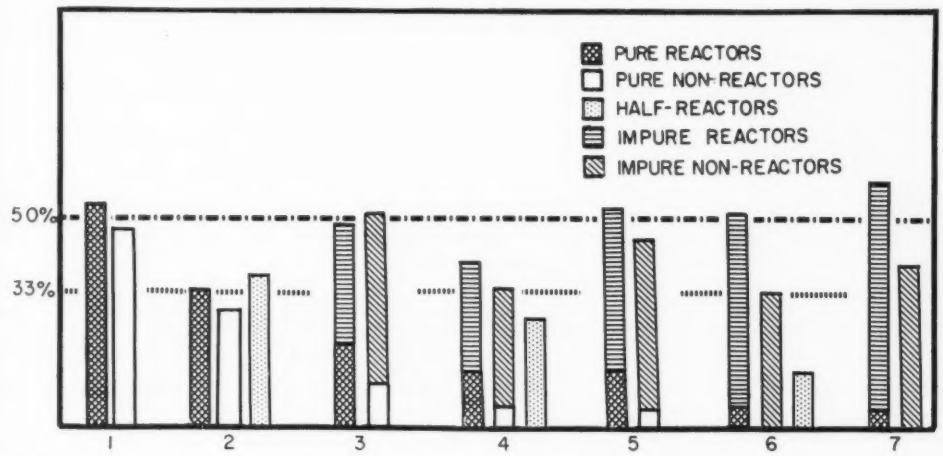


Fig. 6.—The incidence of reactors and nonreactors, pure and impure, and of half-reactors on the basis of 1, 2, 3, 4, 5, 6, and 7 successive placebo tests.

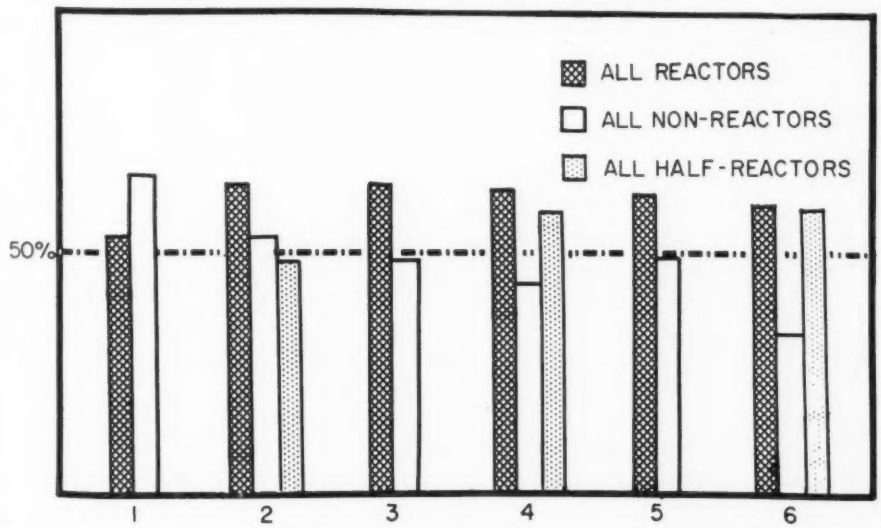


Fig. 7.—The incidence of subsequent placebo responses in the reactor, nonreactor, and half-reactor groups as defined on the basis of 1, 2, 3, 4, 5, or 6 successive placebo tests.

The next question involved an attempt to establish whether or not the occurrence of a placebo reaction had predictive value with respect to the likelihood of that individual displaying further placebo reactions in the future. The

incidence of placebo reactors and nonreactors, pure and impure, and half-reactors on the basis of the first test alone, the first 2 tests, and so on up to and including all 7 tests is illustrated in Fig. 6. There was essentially a 50:50 distribution of reactors and nonreactors when an odd number of tests were analyzed and a 33:33:33 distribution of reactors, nonreactors, and half-reactors when an even number of tests were analyzed. The pure placebo reactor virtually disappeared from the group after the sixth successive test. The observed variations are well within the range of chance, and none differed significantly from the theoretical expected values (Fisher's Exact Method of analysis).

It was not possible on the basis of 1, 2, 3, 4, 5, or 6 tests to predict whether or not an individual would display a placebo response on subsequent testing (Fig. 7). Further, the pure reactors, even when defined on the basis of 5 previous successive placebo tests, showed no greater incidence of subsequent positive responses than when defined on the basis of 1, 2, 3, or 4 previous tests (Fig. 8).

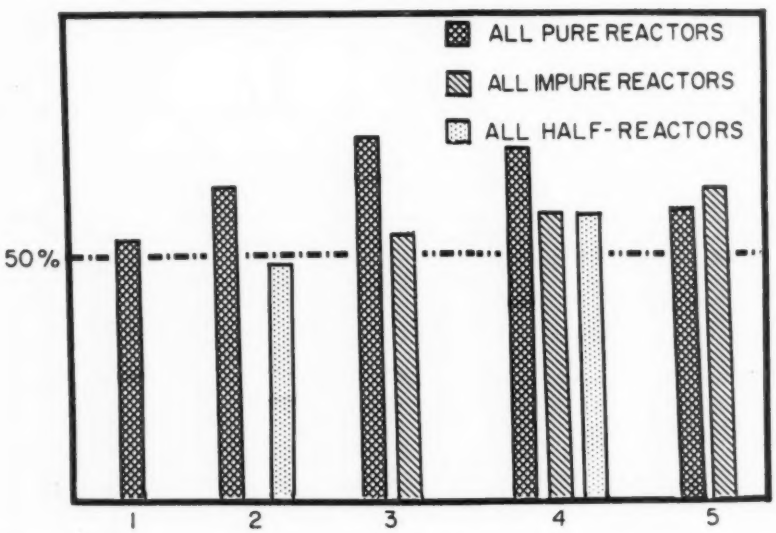


Fig. 8.—The incidence of subsequent placebo responses in the pure reactor, impure reactor, and half-reactor groups as defined on the basis of 1, 2, 3, 4, and 5 subsequent placebo tests.

The incidence of reactors, nonreactors, and half-reactors was examined on the basis of the first test compared to the last test, the first 2 tests compared to the last 2 tests and then the first 3 tests compared to the last 3 tests (Table I). The individual consistency was also examined for each of these groups, comparing the first to the last tests as above. No significant differences occurred and the consistency of responses was nowhere greater than would be expected to occur by chance.

TABLE I

	REACTORS	NONREACTORS	HALF-REACTORS	CONSISTENT RESPONSE
FIRST TEST COMPARED TO LAST TEST				
First	19 (54%)	16 (46%)	0	18 (51%)
Last	18 (51%)	17 (49%)	0	
FIRST TWO TESTS COMPARED TO LAST TWO TESTS				
First 2	12 (34%)	10 (29%)	13 (37%)	11 (31%)
Last 2	15 (43%)	10 (29%)	10 (29%)	
FIRST THREE TESTS COMPARED TO LAST THREE TESTS				
First 3	17 (49%)	18 (51%)	0	20 (57%)
Last 3	22 (63%)	13 (37%)	0	

SUMMARY

These data do not support the concept that either a placebo reactor or a nonreactor really exists as a separate or distinct entity in an experiment measuring an objective phenomenon (vomiting) and a subjective phenomenon (nausea) or even with respect to the potentially more highly suggestible partial relief of nausea and/or vomiting. Since the intraindividual variation in response to a placebo was found to be as great as the interindividual variation, the likelihood of predicting placebo responses was not enhanced by increasing the number of placebo tests performed on any individual.

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THE SALIVARY EXCRETION OF I^{131} IN VARIOUS THYROID STATES

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INTRODUCTION

THAT inorganic iodide is excreted by the salivary glands has been known for a long time,[1] and its high concentration relative to plasma levels has been shown.[2, 3] Ingbar and Freinkel[4, 5] recently demonstrated that the concentration of iodide in the saliva is governed primarily by the level of inorganic iodide in the plasma. Thode and co-workers[6, 7] previously found an inverse correlation between the level of thyroid activity and the concentration of iodide excreted in the saliva.

Since available data on salivary excretion of iodide are still scarce and the subject of renewed interest, the following study of the excretion of radioactive I^{131} in the saliva of patients in various states of thyroid function was undertaken.

MATERIAL AND METHODS

The patients examined were referred to our isotope laboratory for assessment of thyroid function. They were classified on the basis of clinical findings alone as hypo-, eu-, and hyperthyroid. Cases in which the diagnosis was in doubt were classified with the euthyroid. This seems justified by medical considerations and by the assumption that errors due to the inclusion of these cases in the group of euthyroids would tend to cancel out. In most cases studied the clinical and laboratory findings were consistent.

Saliva samples were collected by simple expectoration 24 hours after oral administration of tracer doses of about 200 microcuries (μc) I^{131}, with carrier of less than 1 microgram sodium iodide added. Samples were measured in flat brass planchets, 3 cm. in diameter, after slow desiccation at a temperature below the boiling point of the solution to avoid sublimation of the iodine. A standard solution of 0.001 curies per milliliter of I^{131} was desiccated in a similar manner. The radioactivity of the samples was determined by placing the planchets 3 mm. from the 2 mg./cm.^{2} mica window of a heavily lead-shielded Geiger counter. A relatively high counting efficiency of beta radiation (400 CPM approximately for 0.001 μc) thus was achieved. The method is about 20 times more sensitive than the traditional one using a liquid counter.[8] No correction was made for self-absorption of beta radiation since the thin homogenous layer of dried substance covering the planchet after desiccation never exceeded 30 mg./cm.^{2} Still, a variation up to 30 per cent was found when different planchets, containing identical samples, were compared. This efficient, but somewhat cumbersome, method was developed because a well-type scintillation counter was not available in our laboratory at that time.

Plasma samples were also taken 24 hours after the tracer dose. The free iodide was obtained by precipitation of the protein-bound (PB) fraction with trichloroacetic acid. After the free iodide was decanted, the precipitate was washed and redissolved in 2N

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NaOH.[9] The I^{131} content was expressed as "conversion ratio" and per cent dose per liter plasma. In accordance with the usual methods, the thyroid uptake and urinary excretion of I^{131} were measured 24 and 48 hours after administration of the tracer dose. Geiger counters were used with bismuth- or lead-coated cathodes which improve their sensitivity to gamma rays.

RESULTS

The concentration of radioiodine in the saliva was found to be highest in the hypothyroid state and to decrease steadily as the thyroid activity of the patients increased to hyperthyroid levels. The mean salivary concentration of the hypothyroid group was found to exceed that of the hyperthyroid group by a factor of 20, approximately. Complete separation was found between even the lowest salivary concentrations of the former group and the highest ones of the latter. The mean salivary concentration of I^{131} in the clinical euthyroid group was found in between the values of the two other groups, though some of the individual cases were found within the range of either neighboring section (Fig.

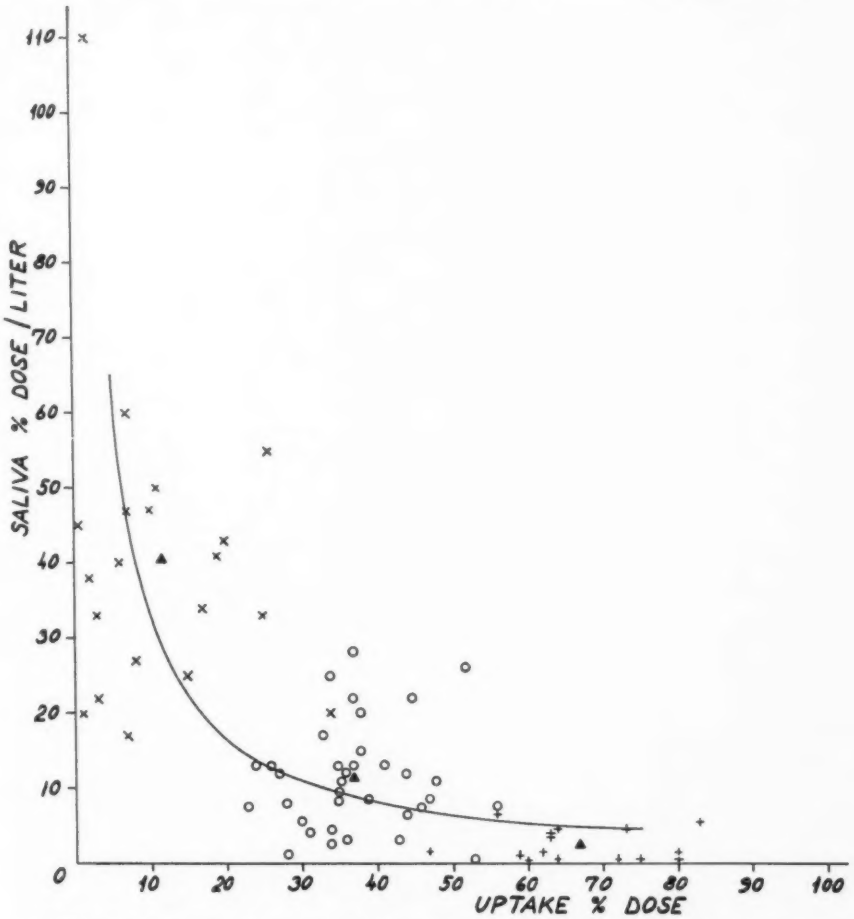

Fig. 1.—Salivary I^{131} concentrations compared to thyroid uptake in various states of activity.

× = Clinically hypothyroid cases.
○ = Clinically euthyroid cases.
\+ = Clinically hyperthyroid cases.
▲ = Corresponding mean values.

TABLE I. DETERMINATION OF I^{131} IN SALIVA, PLASMA, URINE, AND THYROID GLAND

CASE NUMBER	UPTAKE THYROID		EXCRETION IN URINE		SALIVARY CONCENTR. PER CENT DOSE PER LITER	PLASMA CONCENTRATION PER CENT DOSE PER LITER			PB I^{131} CONVERSION RATIO PER CENT OF TOTAL
						TOTAL I^{131}	ORGANIC I^{131}	PB* I^{131}	
	24H	48H	24H	48H	24H	24H	24H	24H	24H
					HYPOTHYROIDS				
1	2	1	77	93	110	0.90	0.81	0.09	10
2	7	3	80	92	60	0.75	0.60	0.15	20
3	26	31	42	50	55	0.30	0.23	0.07	23
4	11	14	68	75	50	0.33	0.25	0.08	24
5	10	9	68	88	47	0.62	0.50	0.12	19
6	7	6	62	78	47	0.90	0.71	0.19	21
7	0.5	0.5	39	51	45	0.52	0.46	0.06	12
8	20	30	40	56	43	0.23	0.22	0.01	3
9	19	21	58	65	41	0.24	0.20	0.04	17
10	6	5	–	–	40	0.33	0.29	0.04	12
11	2	4	50	61	38	0.72	0.69	0.03	4
12	17	19	76	81	34	0.16	0.16	0.00	1
13	3	3	67	87	33	0.92	0.80	0.12	13
14	25	26	61	68	33	0.55	0.44	0.11	20
15	8	6	71	90	27	0.92	0.80	0.12	13
16	15	17	46	52	25	0.40	0.36	0.04	10
17	3	2	89	97	22	0.24	0.20	0.04	17
18	1	0.5	76	78	20	0.47	0.43	0.04	8
19	34	35	49	55	20	0.50	0.46	0.04	8
20	7	4	59	74	17	0.50	0.31	0.19	38
Mean	11.3	12.0	60.4	72.6	40.4	0.59	0.50	0.09	15
					EUTHYROIDS†				
1	37	40	60	64	28	0.42	0.29	0.13	31
2	52	51	30	34	26	0.29	0.25	0.04	14
3	34	37	57	61	25	0.21	0.18	0.03	14
4	37	37	51	55	22	0.22	0.18	0.04	18
5	45	43	51	53	22	0.30	0.17	0.13	43
6	38	41	37	41	20	0.37	0.32	0.05	14
7	33	30	65	67	17	0.35	0.23	0.12	34
8	38	36	–	–	15	0.13	0.10	0.03	23
9	41	40	44	47	13	0.16	0.14	0.02	13
10	24	25	40	45	13	0.17	0.13	0.04	23
11	26	30	63	64	13	0.56	0.46	0.10	18
12	27	31	55	58	12	0.19	0.15	0.04	21
13	36	36	57	61	12	0.17	0.12	0.05	29
14	36	37	50	52	12	0.22	0.18	0.04	18
15	36	41	51	54	12	0.09	0.06	0.03	28
16	44	42	58	62	12	0.29	0.26	0.03	10
17	36	41	55	59	11	0.27	0.21	0.06	22
18	48	41	52	54	11	0.22	0.19	0.03	14
19	35	37	58	60	9.5	0.31	0.23	0.08	26
20	35	35	44	46	8.6	0.14	0.07	0.07	50
21	–	39	47	51	8.6	0.30	0.26	0.04	13
22	47	46	55	58	8.5	0.53	0.45	0.08	15
23	28	31	43	44	8.0	0.40	0.26	0.14	35
24	46	47	42	44	7.5	0.14	0.12	0.02	14
25	56	61	41	42	7.5	0.16	0.13	0.03	19
26	23	25	57	59	7.3	0.37	0.32	0.05	14
27	44	46	44	47	6.5	0.13	0.05	0.08	66
28	30	31	48	50	5.5	0.28	0.20	0.08	28
29	34	33	61	63	4.7	0.45	0.41	0.04	7
30	31	32	47	48	4.0	0.10	0.06	0.04	40
31	36	38	36	38	3.0	0.09	0.05	0.04	67
32	43	43	43	44	3.0	0.09	0.03	0.06	44
33	34	34	43	44	2.7	0.11	0.05	0.06	55
34	28	30	59	60	0.8	0.06	0.05	0.01	25
35	53	53	33	34	0.4	0.15	0.14	0.01	7
Mean	37.0	38.2	49.3	51.8	12.0	0.242	0.187	0.055	26.1

*PB I^{131} = Protein-bound I^{131}.

†Included are cases of clinically doubtful diagnosis.

TABLE I—CONT'D

CASE NUMBER	UPTAKE THYROID 24H	UPTAKE THYROID 48H	EXCRETION IN URINE 24H	EXCRETION IN URINE 48H	SALIVARY CONCENTR. PER CENT DOSE PER LITER 24H	PLASMA CONCENTRATION PER CENT DOSE PER LITER: TOTAL I^{131} 24H	PLASMA CONCENTRATION PER CENT DOSE PER LITER: ORGANIC I^{131} 24H	PLASMA CONCENTRATION PER CENT DOSE PER LITER: PB* I^{131} 24H	PB I^{131} CONVERSION RATIO PER CENT OF TOTAL 24H
HYPERTHYROIDS									
1	56	48	24	25	6.5	0.20	0.05	0.15	75
2	83	66	9	11	5.7	0.65	0.03	0.62	95
3	64	48	20	23	4.7	1.70	0.05	1.65	97
4	73	72	12	13	4.3	0.27	0.02	0.25	93
5	63	67	30	31	4.0	0.32	0.14	0.18	56
6	63	53	30	32	3.6	0.31	0.01	0.30	95
7	47	50	46	48	1.6	0.18	0.07	0.11	61
8	62	55	23	24	1.6	0.58	0.04	0.54	93
9	80	82	18	19	1.4	1.10	0.15	0.95	86
10	59	54	25	26	0.8	0.24	0.04	0.20	83
11	75	71	13	13	0.6	0.70	0.05	0.65	93
12	64	66	16	17	0.5	0.30	0.02	0.28	95
13	72	69	27	28	0.5	1.60	0.24	1.36	92
14	80	76	8	9	0.4	0.50	0.17	0.33	66
15	60	61	21	22	0.1	0.36	0.02	0.34	94
Mean	66.7	62.6	21.4	22.6	2.42	0.60	0.07	0.53	85

TABLE II. MEAN VALUES ILLUSTRATING THE RELATIONSHIP OF SALIVARY I^{131} TO PLASMA I^{131} FRACTIONS*

THYROID ACTIVITY	NUMBER OF CASES	I^{131} SALIVARY CONCENTR. PER CENT DOSE PER LITER	I^{131} PLASMA CONCENTRATION PER CENT DOSE PER LITER: TOTAL	INORGANIC	PROTEIN-BOUND	PB I^{131} CONVERSION RATIO	RATIO OF SALIVARY I^{131}: TOTAL PLASMA	INORGANIC PLASMA I^{131}	PB I^{131}
Hypothyroid	20	40.4	0.59	0.50	0.09	15	69.5	80.5	448
S.E.†		1.5	0.17	0.16	0.02	2	21	26	70
Euthyroid	35	12.0	0.24	0.19	0.06	26	49.6	64.0	216
S.E.		1.2	0.07	0.06	0.02	8	15	22	75
Hyperthyroid	15	2.4	0.60	0.07	0.53	85	4.0	34.5	4.6
S.E.		0.6	0.13	0.02	0.12	12	1.3	13	1.5

*All determinations made 24 hours after tracer dose.
†Standard error.

1). This could be anticipated in a general way and may be accounted for in part by the inclusion of the clinically doubtful cases in the euthyroid group. Graphic correlation of the salivary I^{131} concentrations to uptake in the thyroid reveals an inverse relationship roughly in the shape of a hyperbola. At 24 hours, salivary concentration values above 25 per cent dose per liter were found indicative of hypothyroid state, and those below 5 per cent dose per liter indicative of the hyperthyroid state.

Irrespective of thyroid activity, the ratio of concentrations of I^{131} in the saliva to that of inorganic iodide in the plasma was found to be practically constant, amounting to about 60 (Table II, Fig. 2). The ratio of the concentrations of I^{131} in saliva to that of the protein-bound I^{131} plasma fractions, on the other hand, tends to increase rapidly with increase in thyroid activity.

The total plasma I^{131} concentration at 24 hours was least in the euthyroid group. This can be explained by the fact that most of the radioiodine is either excreted in the urine or stored in the thyroid gland and thus removed from the circulation by 24 hours. The higher total radioiodine plasma values encountered in hyperthyroid states are due to the increased release of radioactive protein-bound iodine into the blood stream,[10] while in hypothyroidism, on the other hand, the high values are due to the delayed urinary excretion of inorganic iodide.[11-13, 15]

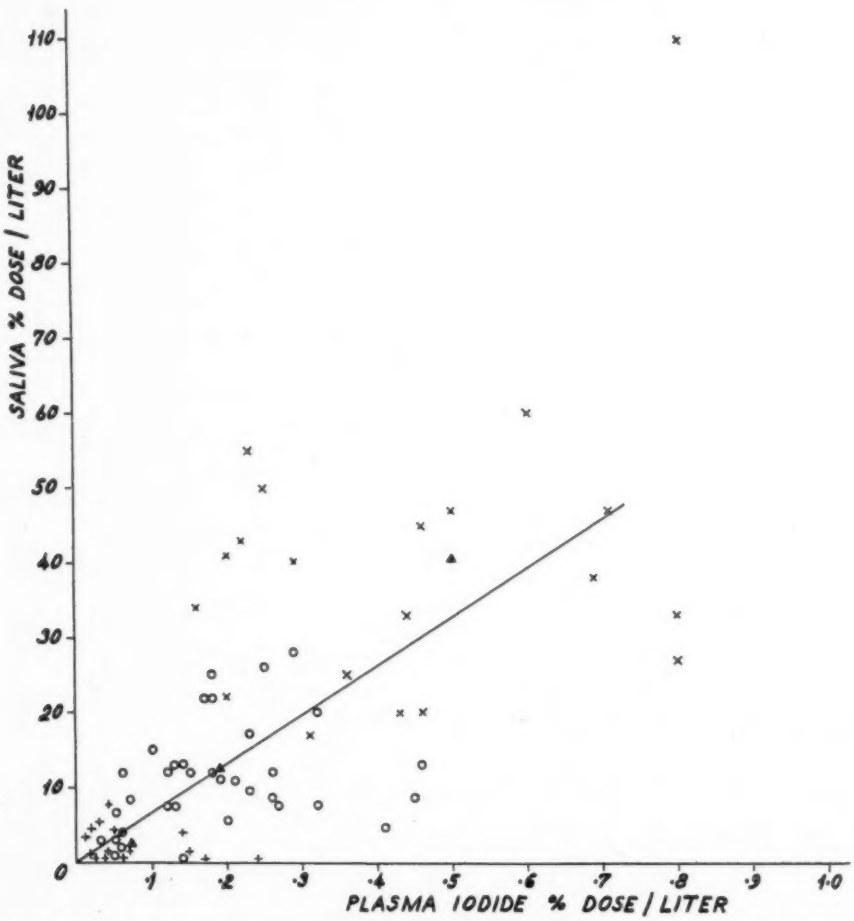

Fig. 2.—Salivary I^{131} concentrations as function of inorganic plasma radioiodide concentrations.
× = Clinically hypothyroid cases.
○ = Clinically euthyroid cases.
+ = Clinically hyperthyroid cases.
▲ = Corresponding mean values.

DISCUSSION

As the concentration of salivary I^{131} is proportional to that of inorganic plasma iodide, as was pointed out, it follows that salivary to total plasma I^{131}

ratio will be highest when free plasma radioiodide predominates and approaches the total plasma concentration. Conversely, a high protein-bound I^{131} concentration will tend to lower the ratio.

This can be expressed easily by the following equations:

$$\frac{S}{F} = C \quad \text{where C is constant, approximately 65 (Fig. 2)} \qquad \text{I.}$$

$$T = F + B \quad \text{by definition} \qquad \text{II.}$$

therefore

$$\frac{S}{T} = \frac{C \cdot F}{F + B} = \frac{C}{1 + \frac{B}{F}} \qquad \text{III.}$$

S = Salivary radioiodine concentration.
F = Inorganic plasma radioiodine concentration.
B = Protein-bound plasma radioiodine concentration.
T = Total plasma radioiodine concentration.

Equation III shows, in fact, that the salivary to total plasma I^{131} ratio approaches the constant C as B, the protein-bound plasma radioiodine concentration, decreases toward zero. The ratio approaches zero as F, the inorganic plasma radioiodide concentration, decreases toward zero.

While the mean values obtained experimentally as given in Table II fit in well in this equation, it is doubtful whether the ratio can be applied successfully for diagnosing individual cases, as is apparent from the considerable scatter of the individual values in Table I and Fig. 2. Considering the basic physiologic relationship between the inorganic plasma I^{131} and the salivary I^{131} which is practically independent of thyroidal function, one may expect closer agreement of the respective values of salivary I^{131} concentrations to total plasma concentrations with improvement of procedure, chiefly toward uniformity of saliva collection. However, since salivary I^{131} concentration has been shown to be roughly inversely related to salivary flow,[5, 13, 14] it is doubtful to what extent error can be eliminated since variations in salivary flow are inevitable, thus distorting the characteristic saliva to plasma ratio considerably. Incidentally, our experimentally obtained deviation from constancy of the salivary to inorganic plasma I^{131} concentration ratio, 80.5 in the hypothyroid, 64 in the euthyroid, and 34.5 in the hyperthyroid (Table II), may be due to variations in salivary flow rates in diverse thyroidal states.

Another source of error should be kept in mind when salivary tracer findings are interpreted. In the rather frequent condition of kidney impairment, salivary I^{131} concentration will be misleading in the assay of thyroid function. In such cases, inorganic iodide will be retained for longer than usual periods in the blood, owing to decreased renal clearance. This will in turn increase I^{131} concentration in the saliva at 24 hours although protein-bound I^{131} concentration will be of normal magnitude and will seem relatively diminished in comparison to the inorganic plasma fraction. Whereas the handling of a tracer dose is generally reflective of iodine metabolism in the body, this rule will not hold true in the presence of renal impairment.

The determination of salivary to protein-bound plasma I^{131} ratio as proposed by Thode and co-workers[6] cannot be regarded as reflective of any intrinsic biologic relationship, as salivary I^{131} excretion is unrelated to thyroidal function and determined by plasma inorganic I^{131} level alone. Still, it may prove of considerable diagnostic merit in tracer examination by providing marked differentiation of thyroid states. This differentiation is achieved by introducing the salivary iodide concentrating mechanism, thus comparing the augmented salivary I^{131} value, instead of the total plasma I^{131} value as used in the conventional PB I^{131} conversion ratio test, with the PB I^{131} plasma fraction. Without foregoing this diagnostic advantage, the method of comparing salivary to total plasma I^{131} concentrations is preferable to the conventional method of measuring the PB I^{131} conversion ratio for several practical reasons: (a) the ease of counting the relatively high salivary I^{131} concentrations, (b) the relative simplicity of saliva collection, and (c) the higher accuracy obtainable when dealing with total plasma alone as compared to the two plasma fractions which are not easily separated from one another entirely in routine laboratory procedure, and the time thus saved.

SUMMARY

The salivary radioiodine excretion in various functional thyroid states was examined in 70 patients 24 hours after administration of a tracer dose. In each patient, the concentration of inorganic and protein-bound plasma radioiodine was measured as well as thyroid uptake and urinary excretion of I^{131}.

High salivary I^{131} concentrations were consistently found in hypothyroid subjects, decreasing as thyroid activity increased. Salivary I^{131} concentration varied considerably in clinically euthyroid patients, though the mean values obtained in each of the functional states were distinctly apart.

A practically constant ratio was found between the mean values of salivary I^{131} and plasma inorganic I^{131}, regardless of thyroid state of the patients, furnishing further evidence of the biologic relationship of these two entities. Though appropriate for diagnostic purposes, the salivary to protein-bound plasma I^{131} ratio does not reflect any intrinsic biologic relationship.

The determination of both salivary and total plasma I^{131} concentrations may be satisfactory for estimation of thyroid activity by tracer methods. It is more convenient from practical considerations than the usually practiced determination of PB I^{131} conversion ratio and may furnish equivalent diagnostic information.

Sources of error with special regard to variation of salivary flow rate as well as limitations inherent in the method are discussed.

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STUDIES OF EXPERIMENTAL PULMONARY EMBOLISM AND INFARCTION AND THE DEVELOPMENT OF COLLATERAL CIRCULATION IN THE AFFECTED LUNG LOBE

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GENERAL knowledge of the genesis of pulmonary infarction has been gained from numerous clinical and experimental studies. It has been widely held that lobar embolism of the lungs does not usually cause infarction unless congestion, infection, or ventilatory disturbances are also present. It has been assumed that any condition interfering with the blood supply of the lung tissue will aid the formation of infarcts resulting from pulmonary arterial obstruction by inhibition of the development of collateral circulation.[1-5] However, several recent studies have suggested alternative mechanisms.[6-8] The precise physiologic events in lung infarction have remained in doubt.

We have observed that the embolic obstruction of the lobar arteries of dogs with established arteriovenous fistulas regularly induced infarction of the affected lobes. This method of producing pulmonary infarction appeared to be preferable to those entailing surgical manipulation of the lungs or the use of noxious agents such as alpha-naphthylthiourea. Our work was undertaken with the aim of clarifying the processes of lung infarction in relation to the collateral circulation which may develop in the injured area.

METHODS

Mongrel dogs of varying weight (5 to 20 Kg.) were used. Under Nembutal anesthesia, unilateral or bilateral femoral arteriovenous fistulas were constructed by side-to-side anastomosis in 29 animals. The average length of the stoma was 2.5 cm. Immediately after the fistulas were completed, pulmonary embolism was produced by the introduction of one or two (2 cm.) segments of rubber urethral catheter (No. 6 or 8 Fr.) into an external jugular vein. In some instances embolization was carried out with aluminum cylinders 1.5 × 0.5 cm., and occasionally with slightly larger caliber rubber tubing. In 8 dogs, pulmonary embolism was performed after the fistulas had functioned for 5 to 180 days. Twenty animals, without arteriovenous shunts, were subjected to similar lung embolism for control observation. Each animal received 400,000 units of penicillin and 0.25 Gm. of streptomycin on completion of the operations. The dogs were killed from 23 hours to 58 days (average 7 days) after embolization, and the lungs were examined for evidence of infarction grossly, and in some cases microscopically.

In order to assess the extent of the lobar collateral circulation following embolism, 17 of the control and infarct animals were studied to quantitate the blood flow in the affected lobe, using methods similar to those of Shedd and associates[7] and of Williams and

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Towbin.[9] Under anesthesia, the chest was opened by sternal incision, and the animal was heparinized. The vein of the embolized portion of the lung was cannulated so as to obtain all of the lobar venous blood. The corresponding lobar artery was ligated proximal to the lodged embolus, which was easily palpable. Under these conditions, the collected blood was assumed to originate from the bronchial arterial flow into the lobe.[7, 9] Mean carotid arterial blood pressure was recorded by a simple mercury manometer. Blood flowing from the lung lobe was returned to the animal by venous infusion.

To observe the possible anatomical changes in the bronchial arterial vasculature, the lungs of normal animals, those with emboli, and those with obvious infarcts were studied by neoprene latex* injection and corrosion.[10] The procedure, similar to that of Cockett and Vass,[11] was done with open thorax. Immediately after exsanguination and death, a large glass cannula was inserted into the brachiocephalic artery for injection of the aorta and its branches. The left subclavian artery was ligated, and the aorta was tied immediately superior to the diaphragm and also above the origin of the coronary vessels. A second large cannula was introduced into the main pulmonary artery through a right ventricular incision and secured by ligatures. An opening in the left ventricular wall permitted escape of the perfusion fluid. Saline was then perfused under pressure until the escaping fluid was grossly clear of blood. The aorta and its tributaries were injected with red neoprene (colored by the addition of Du Pont Red PBL as a 25 per cent dispersion in 1 per cent NaOH), at 250 to 300 mm. Hg pressure for 3 to 5 minutes. Satisfactory injection of the aortic branches was signaled by visible filling of the intercostal arteries with latex. Injection of the pulmonary artery was carried out with blue-colored latex (Du Pont Blue CPL) at a pressure of 100 mm. Hg. The lung tissue assumed a blue color. Four per cent acetic acid was then poured into the thoracic cavity to coagulate any escaping latex, and the thoracic viscera were removed in toto. Yellow latex (Du Pont Yellow GL) was poured into the trachea to fill the larger air passages to give added support to the finished casting. The specimen was placed in a mixture of 4 per cent acetic acid and 10 per cent formalin for 18 hours to fix the tissue and to coagulate the rubber. Thereafter the heart, pericardium, esophagus, and aorta were trimmed away, and the specimen was immersed in concentrated hydrochloric acid for 48 to 72 hours. The acid and digested material were displaced by running tap water, and the rubber casting of the lung vessels was kept immersed for study and photography.

RESULTS

Infarction of the Lungs.—In animals with functioning arteriovenous fistulas, the nearly constant occurrence of infarction following embolism was striking (Table 1). Of the 29 fistula dogs subjected to lung emboli, 24 showed gross evidence of lung infarction as judged by consolidated, hemorrhagic and necrotic areas of tissue distal to the palpable foreign body embolus (see also Fig. 1). In 3 of the dogs there were minor changes in the parenchyma, and in 2 other animals with identifiable emboli there was no evidence of lung damage. None of the dogs developed gangrenous necrosis of the pulmonary parenchyma.

TABLE I. COMPARISON OF THE CONTROL DOGS AND FISTULA DOGS FOLLOWING PULMONARY EMBOLISM WITH FOREIGN BODIES

TYPE OF EXPERIMENT	NUMBER OF DOGS	DOGS WITH INFARCTS	NO INFARCTS	MINOR LUNG CHANGES
Control dogs	20	1	17	2
Fistula dogs	29	24	2	3

*Neoprene latex was generously supplied by E. I. Du Pont de Nemours & Company, Wilmington, Del.

Quite in contrast, of the 20 dogs *without* arteriovenous fistulas which were subjected to pulmonary embolism, only one developed an infarct of the lung. This dog was ill of an apparent neurotrophic virus infection, and the formation of the lesion may have been favored by a subtle pneumonic process or by lung congestion.

The small random emboli most frequently lodged in the lower lobar arteries, especially the left. The embolic effects were the same regardless of the type of foreign body (rubber or metal) or the location of the embolus in the lobar artery. It was also notable that the *duration* of the fistulas before pulmonary embolism was carried out did not influence the incidence of lung infarction: e.g., pulmonary arterial obstruction within a few minutes after the establishment of an arteriovenous communication resulted in infarction as often as embolism in dogs with a shunt of longer standing. It is also of importance that the incidence and character of the pulmonary infarcts were the same whether the dogs had unilateral or bilateral fistulas. In fact, the presence of only one arteriovenous shunt was sufficient to permit serious lung injury by the embolic closure of a lobar artery.

The fistula animals which survived 4 to 8 weeks after embolism usually exhibited resolution of the lung infarcts. The affected areas became partially fibrotic and shrunken; hemorrhage and consolidation disappeared, and reaeration of the alveoli occurred.

Collateral Blood Flow Before and After Pulmonary Embolization.—In open-chest preparations with normal lungs, the magnitude of bronchial arterial blood flow was similar to that previously reported.[9] The collateral blood flows are shown in Table II. In control dogs without previous lung embolism, the quantity of blood recovered from a lobar vein was small (1 c.c. or less per minute). However, as the time interval (4 to 7 weeks) following arterial obstruction increased, there was striking augmentation of collateral flow. Nevertheless, individual variations in outflow were marked. The amount of bronchial collateral flow reached as high as 16 to 26 ml. per minute in some cases. These experiments confirmed the results of Williams and Towbin[9] which indicated that increase in lobar outflow occurred within a few days after the pulmonary artery had been occluded. In accord with their findings, our experiments showed that the collateral outflow varied directly with systemic arterial blood pressure (Table II).

It was of interest that the bronchial collateral flow in the fistula animals with pulmonary infarction was likewise strikingly increased in 4 to 7 weeks. Indeed, the highest flow values noted were in the fistula dogs. Therefore, it is evident that the stimulus to the enlargement of the collateral circulation exists whenever occlusion of the lobar arteries occurs, whether peripheral arteriovenous shunts are present or not. It is clear that, in these instances, infarction must be related to other factors or conditions which annul the effects of the increase in collateral channels, so that breakdown of the parenchyma ensues.

TABLE II

DOG	WEIGHT (KG.)	LOBE WITH EMBOLISM	TIME AFTER EMBOLISM	FISTULA PRESENT	AVERAGE B. P. (MM. Hg)	PULMONARY LOBAR VEIN FLOW (C.C./MIN.)	INFARCT	REMARKS
3	15.9	LLL	minutes	+ acute	85	less 1.0	0	Fistula with tubing
14	7.2	LLL	minutes	0	65	less 1.0	0	
15	8.1	LLL	minutes	0	100	less 1.0	0	
8	10.4	LLL	minutes	0	80	1.0	0	
4	20.0	LLL	minutes	+	110	6.0	0	Fistula with tubing
11	14.5	RLL	9 days	+	80	6.0	+	Chronic fistulas (2)
12	27.2	RML	11 days	+	80	8.0	+	Chronic fistulas (2)
75	8.1	LLL	28 days	+	85	29.0	+	Fistula closed few days after embolus
72	9.0	LLL	39 days	+	90	6.0	+	Fistula was transient
60	18.6	LLL	40 days	+	70	38.0	+	
69	15.0	LLL	47 days	+	80	22.0	+	
53	11.8	LML	48 days	0	75	6.0	0	
64	10.0	LLL	48 days	0	110	16.0	0	
65	9.0	LLL	49 days	0	75	16.0	0	
55	10.0	RLL	49 days	0	60	26.0	0	
54	7.2	LLL	52 days	0	70	3.0	0	
51	7.7	LML	63 days	+	65	1.0	+	Chronic fistula

Neoprene Latex Injection and Corrosion Preparations.—The normal lungs, after latex filling and corrosion, exhibited the fine tracery of the bronchial arteries extending along the bronchi and the finer bronchial branches until they were lost as delicate threads near the alveoli (Fig. 2). Careful inspection of the castings frequently showed minute precapillary anastomoses, appearing as arcades, between small and medium-sized bronchial and pulmonary arteries. Such interarterial communications have been previously described.[3, 11, 12] Study of the lungs of dogs which had been subjected to pulmonary embolism 8 days or longer before latex injection showed that the bronchial arteries had increased vastly in size (Fig. 3). Pronounced dilatation of the bronchial-pulmonary arterial anastomoses evidently occurred, for red latex from the aorta penetrated and completely filled the pulmonary arterial system of the involved lobe. It seems probable that the integrity of the lung tissue may be maintained by this collateral circulation, even with the lobar artery completely obstructed.

In the fistula animals with lobar embolism and frank pulmonary infarction, neoprene injection of the bronchial arteries likewise revealed an extensive filling within the infarcted tissue from the bronchial arterial system (Figs. 4 and 5). The collateral circulation in these instances seemed to be fully as great as in the embolized, but noninfarcted lungs. Furthermore, as in the case of the control dogs with emboli, the development of the increased bronchial arterial circulation required a number of days (apparently 8 or more) to occur. It was also found that with pulmonary arterial injection of neoprene there was no instance of filling of the pulmonary arterial tree distal

to the point of impaction of the embolus, in spite of the rather high injection pressures used (Fig. 6). Therefore, any latex which entered the lobar tissues was clearly derived from the aortic connections.

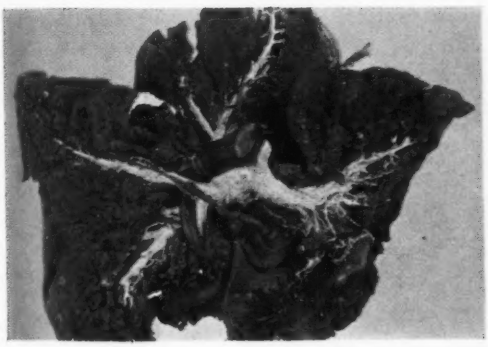
DISCUSSION

Although experimental pulmonary embolism has received extensive study, less attention has been given to the mechanism of pulmonary infarction. It is known that simple ligation or embolization of a pulmonary artery will not usually provoke infarction.[13-15] However, Steinberg and Mundy[16] reported infarction of the lungs in a few instances following multiple lead shot emboli. They believed that total occlusion of lobar arteries alone was necessary for the formation of infarcts.

Most investigators have found it necessary to alter the circulatory dynamics of the lungs in order to induce pulmonary necrosis by embolism. Karsner and Ash[2] produced infarction after embolization when circulatory stasis was caused by ligation of lobar veins or by artificial pleural effusions. Mathes and associates[17] noted hemorrhagic pulmonary consolidation and gangrene from the introduction of infected emboli. Their observations suggested that infection of the lungs interfered with the blood supply of the tissue, and that the dilatation of the bronchial arterial channels aided tissue repair. In a study of lung infarcts in rabbits, Moses[5] bound the chests tightly so as to produce stasis within the lungs; embolism nearly always provoked extensive necrosis. Chapman and his co-workers,[4] using blood clots as emboli, were able to produce pulmonary infarction in dogs when intense congestion was previously induced by alpha-naphthylthiourea. Roach and Laufman[1] made the interesting observation that infarction may result from the impaction of unsterile cotton plugs in the bronchi together with embolism of the lung segment. Embolism in dogs with distemper likewise induced infarction.

The ordinarily held concept that a supplementary circulation can be provided by the bronchial arterial system in the wake of pulmonary arterial obstruction is direct and logical.[18] According to this concept, pulmonary engorgement, infection, or decreased ventilation[1, 19] leads to intrapulmonic circulatory stasis which impedes irrigation through the connecting bronchial arteries and nullifies the benefits of the collateral blood supply. In contrast, Ellis, Grindlay, and Edwards[6] and Shedd, Alley, and Lindskog[7] have suggested that adequate function of the bronchial arterial system encourages the formation of infarcts when embolism is sustained. Ellis and associates showed that occlusion of the bronchial artery to the right upper lobe of dogs preceding multiple glass bead emboli did not result in infarction, although occasional infarcts occurred in other undisturbed lobes which were more heavily embolized. They also noted that pulmonary emboli caused infarction when the corresponding lobar veins were ligated, whether or not the bronchial artery to the part had been occluded. These results implied that the bronchial arteries are not essential to the sustenance of the involved area. They postulated that

Fig. 4.



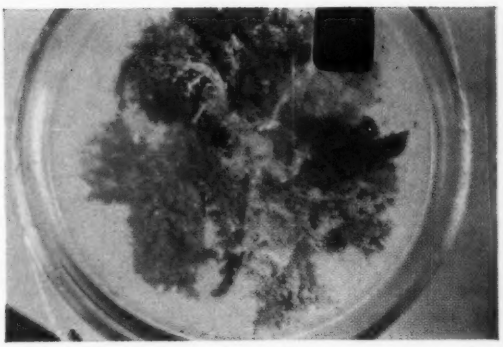
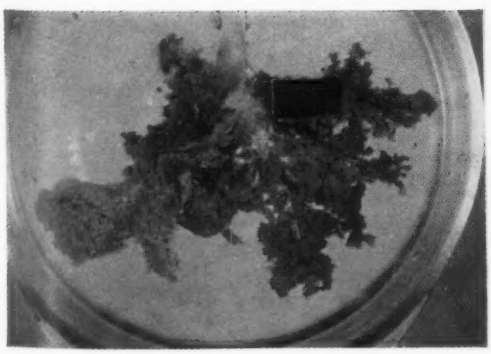


Fig. 5.



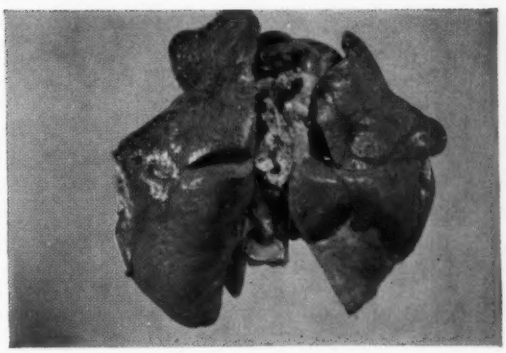


Fig. 6.

Fig. 1.—Characteristic gross appearance of a pulmonary infarct in a dog with peripheral arteriovenous fistula and pulmonary embolism produced by catheter segment introduced into a vein.

Fig. 2.—Neoprene latex corrosion specimen of normal dog lungs showing the pulmonary arterial vasculature (blue), bronchial arteries (red), and tracheobronchial tree (yellow).

Fig. 3.—Latex-corrosion specimen of dog lung 2 weeks after catheter-segment embolization. No arteriovenous fistula and no evidence of pulmonary infarction. Absence of blue latex in lower lobe indicating complete pulmonary arterial obstruction. Red latex injected via the aorta fills the vasculature of the embolized lobes by way of bronchial arterial collaterals. Contrast with Fig. 2.

Fig. 4.—Latex injection of lungs of a dog with arteriovenous fistula 28 days after embolism of both lower lobes. Gross evidence of infarction of left lower lobe (to right in picture); opposite lobe showed no infarct. Each embolized lobe shows comparable filling of pulmonary vasculature with red neoprene from bronchial arterial collaterals.

Fig. 5.—Lung specimen of a dog with arteriovenous fistula 7 days after embolism and infarction of right lower lobe. Red neoprene injected into aorta has passed into the pulmonary vascular spaces via bronchopulmonary arterial anastomoses.

Fig. 6.—Gross appearance of lungs (viewed posteriorly) of a dog with embolus in right lower lobar artery. No evidence of infarction. No anteriovenous fistula. Absence of blue neoprene, injected into the pulmonary arteries, distal to the embolus.

pulmonary artery intercapillary anastomoses form the greater part of the collateral circulation. Therefore, the delivery of blood under high pressure to the fine pulmonary arterial vasculature through the bronchial system could overload and damage the alveolar capillaries, with resultant hemorrhage and necrosis. Nevertheless, in their provocative report, the question arises whether critical pulmonary arterial occlusion was obtained with the small number of emboli which reached the right upper lobe. Moreover, it seems possible that obstruction of the lobar veins may invoke severe stasis in those lobes, so that adequate collateral circulation could not function regardless of the previous state of the collateral channels.

It has been difficult to ascertain the precise mechanisms by which arteriovenous fistulas alter the pulmonary vascular dynamics to enhance infarct formation. Our data do not indicate that collateral flow provided by pulmonary intercapillary anastomoses is important in preventing the lesion. In fact, lung infarction occurred nearly as frequently when the emboli were deeply lodged in the lobe (where pulmonic interarterial anastomoses should be numerous) as when the entire lobar artery was blocked. None of our animals with arteriovenous fistulas exhibited congestive failure or lung engorgement. Hence, general pulmonary vascular stasis was apparently not concerned in the process. The small elevation of right atrial pressure which has been reported to occur with arteriovenous shunts[21, 23, 27] seemed to be of no significance in reducing bronchial arterial flow in our experiments. Therefore, the mechanism of the diminution of bronchial collateral blood supply must have been related to the abnormal peripheral circulation produced by the arteriovenous communication.[20] It is known that bronchial flow decreases with decline of systemic mean and diastolic blood pressures,[9] and declinations of blood pressure occur with the opening of an arteriovenous communication.[20, 24] The possibility is raised in our experiments that the collateral flow in the infarcted area may have been materially lessened by this means. In addition, the free shunting of blood through the low-resistance fistula may produce a compensatory vasoconstriction in the systemic vascular system.[23, 26, 28] It is possible that this vasoconstriction could involve the bronchial arteries as well, thereby dissipating partially their effectiveness as collateral channels. While the question is problematic, it seems credible that these mechanisms may interfere sufficiently with the ancillary vasculature, after pulmonary obstruction, to encourage tissue deterioration and infarct formation.

It is concluded that, when lobar pulmonary arterial obstruction occurs, the intact bronchial arterial vessels are small but adequate to prevent lung infarction when the circulation is otherwise normal. However, if an arteriovenous fistula is present in the systemic circulation at the time of pulmonary embolism, flow through the small bronchial arteries is held at lower values by the mechanisms described before, and the collateral flow becomes inadequate to prevent gradual necrosis of the lung tissue. Nevertheless, a gradual dilatation of the bronchial arteries takes place, following the stimulus of lobar

arterial occlusion, whether or not the fistula is present. This vascular dilatation partially overcomes the tendency to lowered bronchial flow resulting from a fistula to the extent that fibrosis of the necrotic area occurs. Under these conditions, measured collateral bronchial flow is comparable in embolized lobes with or without tissue infarction. From the clinical standpoint, it seems possible that lung congestion from any cause, ventilatory disturbances or infection may derange the *function* of even an anatomically extensive collateral circulation when lobar embolism has occurred, with infarction of the lung as the outcome.

SUMMARY

1. It is possible to produce pulmonary infarction regularly by the use of foreign body emboli in dogs with peripheral arteriovenous fistulas.

2. Dogs with normal circulation infrequently develop infarction of the lungs following pulmonary embolism. It has been shown that the bronchial arterial circulation to the embolized lobe becomes greatly increased, possibly protecting the tissue following embolic obstruction. In "fistula dogs" which have sustained pulmonary infarction after embolization, the development of marked enlargement of the bronchial arterial system is entirely comparable.

3. The evidence suggests that the effects of the bronchial arterial collateral system are offset acutely in the presence of a functioning arteriovenous shunt in the systemic circulation, permitting partial necrosis and eventual fibrosis of the injured lung. However, the collateral circulation is sufficiently large to prevent gangrenous necrosis.

4. We could find no evidence that pulmonary arterial intercapillary anastomoses contributed significantly to the formation of a collateral blood supply in embolized lung parenchyma.

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STUDIES ON EXTRAHEPATIC CHOLESTEROL SYNTHESIS AND EQUILIBRATION IN MAN USING A DOUBLE LABELING TECHNIQUE

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INTRODUCTION

THE primary purpose of this study was to develop a double isotopic labeling technique for the investigation of the metabolism of sterols and steroid hormones in man. As a first application, we have investigated the extrahepatic synthesis of cholesterol and have concurrently obtained data on the relative rates of equilibration of cholesterol between plasma and certain extrahepatic tissues.

Previous studies in experimental animals have shown that the cholesterol present in most organs and tissues may be derived from any one or all of 3 sources, synthesis in the tissue itself, or transport to the tissue via the blood, either of cholesterol synthesized in liver or of that absorbed from the diet.[1, 2] It appears probable that under normal circumstances all 3 sources are utilized. By the use of dietary cholesterol labeled with one radioactive isotope and of acetate (a precursor in the biosynthesis of cholesterol) labeled with a second isotope, it is possible to distinguish between the various potential sources of tissue cholesterol in a single subject.

In the studies reported in the present paper we have fed tritium-labeled cholesterol for periods ranging from 5 to 9 days at such a rate as to maintain an approximately constant plasma cholesterol specific activity to a series of 4 patients scheduled for surgical procedures. The specific activity of cholesterol isolated from tissue biopsies permits a rough estimate of the extent of equilibration between plasma and the tissue concerned.

Estimates of the relative rates of cholesterol synthesis in various tissues were made by use of C^{14}-carboxyl-labeled acetate. When this metabolite is administered in a small dose, it is distributed throughout the whole body and metabolized with great rapidity. In humans, approximately 40 per cent of a 200 μc dose of 1-C^{14}-acetate has been recovered in the expired CO_2 during the 4 hours following an intravenous injection[3]; a small fraction is incorporated

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into cholesterol in liver and in many other tissues. When a tracer amount of acetate is injected, the incorporation process has been found to be complete in mice in 10 to 15 minutes, in dogs in 1 hour, and in man in 2 to 4 hours.[4, 5] In the present study, the time of sampling was chosen to allow for completion of the conversion of C^{14}-acetate into cholesterol, but to minimize the possibility of transfer of C^{14}-cholesterol from liver to extrahepatic tissues. It was based on previous findings concerning the time-course of the plasma free cholesterol specific activity following an intravenous dose of 1-C^{14}-acetate.[3] If free cholesterol isolated from a tissue biopsy taken 1 to 2 hours after C^{14}-acetate administration is found to have a higher specific activity value than plasma, it may be assumed that cholesterol synthesis has occurred in this tissue. A tissue value which is appreciable but lower than the plasma free cholesterol value may be due to synthesis in situ, rapid interchange with plasma cholesterol, or both. Evidence concerning the rapidity of equilibration, which is obtainable from the dietary H^3-cholesterol results, may help to establish whether or not cholesterol synthesis occurs in a specific extrahepatic tissue by putting an upper limit on the amount of C^{14}-cholesterol that could have been carried to the tissue by the blood. In vitro studies can provide a more rigorous proof of the capability of a given tissue for cholesterol synthesis, but this in vivo flash-labeling method, when properly controlled, may give a truer picture of the relative rates of synthesis in various tissues in the living body.

The advantages of using both types of isotopic tracers in a single study include the elimination of the variations in different patients or even in one patient at different stages of a severe or terminal illness and the fact that considerably more information may be obtained from the same number of blood and tissue samples. Methods for counting both C^{14} and tritium in the same sample by means of a liquid scintillation counter, which were previously developed for animal experiments,[6] were utilized in this study.

EXPERIMENTAL METHODS

Patients.—Case 1, Unit No. 568508, a 60-year-old white woman, entered the Argonne Cancer Research Hospital for bilateral adrenalectomy and oophorectomy as a palliative procedure for metastases of a carcinoma of the breast. She had had a radical mastectomy 2 years previously and had since then had been given x-ray therapy and estrogen therapy which had resulted in little objective benefit. A subcutaneous metastasis had been noted in the right chest 21 months before admission, which had broken down with the formation of an ulcer. X-ray examination disclosed no evidence of bone, or other, metastases.

Case 2, Unit No. 571818, a 47-year-old white woman was admitted for bilateral adrenalectomy and oophorectomy. She had had radical mastectomies, both right and left, repeated x-ray therapy, and hormonal therapy during a 4-year period. On admission, she was found to have widespread metastases of almost all ribs, scapula, bony pelvis, proximal femur, lumbosacral vertebrae, and skull; during surgery, metastases of the lower ilium, uterus, peritoneum, and liver were noted.

Case 3, Unit No. 580097, a 36-year-old white woman was admitted for bilateral adrenalectomy and oophorectomy. She had had a radical mastectomy 2 years previously, followed by x-ray therapy. Menstruation had continued until shortly before the operation, at which time she was being given testosterone propionate (300 to 500 mg. per week for the 7 weeks preceding the operation). X-ray examination showed a few osteolytic metastases in the bony pelvis.

Case 4, Unit No. 400342, a 42-year-old white woman was admitted to the Chicago Lying-in Hospital* for complete hysterectomy and oophorectomy for frequent, excessive uterine bleeding. The uterus was of normal size and mobility. The left ovary contained a corpus luteum of approximately 7 to 8 days of age. No ACTH was given prior to the operation; otherwise, the schedule of events was quite similar to that of Case No. 1.

Materials.—C^{14}-carboxyl-labeled acetate with a specific activity of 1 mc. per millimol of acetate, obtained from a commercial source, was used. It was sterilized as previously described[3] and injected intravenously after the patient was anesthetized and before the surgical procedure was started. Closed anesthesia was used and consequently all the $C^{14}O_2$ exhaled by the patient was absorbed in soda lime. The time interval between acetate administration and the removal of each tissue and blood sample was recorded. No blood transfusions were given until after the last biopsy had been taken.

Tritium-labeled cholesterol was prepared by a modification of the method described by Fukushima and Gallagher[7] for deuterium-labeled cholesterol. Because of the high specific activity of the tritium water (H^3OH), it was not necessary to label the solvents. In a typical run 15.0 Gm. of cholesterol acetate was shaken with a mixture of 65 ml. of glacial acetic acid, 1.5 Gm. of freshly reduced platinum catalyst (Adams'), and 19.5 ml. of H^3OH with a specific activity of about 0.3 curies per milliliter, for 3 days at about 130° C. in a sealed flask. The yield was 64 per cent of material with the same melting point as purified cholesterol (147° to 148° C.), and with a specific activity of 1.26 μc per milligram. The material used in these studies had a specific activity of 0.26 μc per milligram (Cases 1 and 2) and 1.08 μc per milligram (Cases 3 and 4).

Determination of the isotope distribution indicated that approximately 47 per cent of the tritium was attached to the side chain and 53 per cent to the ring system. The details of this analysis will be given elsewhere.[8]

The H^3-cholesterol was administered in olive oil solution in gelatin capsules. The amounts of both oil and cholesterol in any single dose were very small, varying from 5 to 50 mg. of sterol and 0.5 to 5 ml. of olive oil. A priming dose was given to bring the plasma H^3-cholesterol specific activity up to the equilibrium range as rapidly as possible. The first 2 patients were given priming doses 10 times the size of the daily maintenance dose, but later it was found that a fivefold priming dose was sufficient.

BIOCHEMICAL METHODS

Analyses for total or free and esterified cholesterol in plasma and tissues were done as prescribed previously[2]; cholesterol was isolated and purified as the digitonide and counted either in this form or after regeneration of the sterol by the pyridine-ether method.[9]

RADIOASSAY METHODS

The methods used for radioassay of C^{14}-labeled cholesterol using either a Geiger counter or a liquid scintillation counter were described in a previous report.[3] In the present study, all samples were assayed for C^{14} with a thin end window Geiger counter and then assayed for both C^{14} and H^3 in a liquid scintillation counter of the type described by Hiebert and Watts.[10] It was found possible to obtain counts for each of the 2 isotopes in the same sample by counting at 2 settings of the pulse height discriminators, at 2 settings of the high voltages on the photomultiplier tubes, or at 2 settings of the 2 amplifier gains. The first of these methods was used in these studies; more recently the third method has been found to have the lowest background for C^{14} counting and the smallest variation with fluctuations in the line voltage. Whichever method of discrimination is used, 2 settings are found, the first to give a zero or very low efficiency for tritium with a reasonably high efficiency for C^{14}, and the second an optimal efficiency for tritium with a minimal for C^{14}. Backgrounds and counting efficiencies for both tritium and C^{14} standards for both settings are determined daily or oftener. The actual counting rates for each isotope are determined by 2 simultaneous equations, if the tritium efficiency at the C^{14} setting is not negligible. Ordinarily, the tritium efficiency is less than 0.03 per cent at the C^{14} setting so that the

*We are greatly indebted to Drs. M. E. Davis and E. J. Plotz of the Chicago Lying-in Hospital for their interest and cooperation in this study.

C^{14} activity may be determined directly. The tritium activity may then be determined from the total counting rate at the tritium setting by subtracting the calculated C^{14} contribution.

The C^{14} standard used was C^{14}-carboxyl-labeled benzoic acid prepared from a $BaC^{14}O_3$ sample* which had been standardized in a mass spectrometer. The H^3 working standard used was a sample of H^3-dihydrocholesterol prepared from cholesterol by hydrogenation with H_2 gas in the presence of H^3OH. It was standardized against a primary H^3OH standard* by direct comparison in a solvent system composed of 90 per cent toluene and 10 per cent of a mixture of absolute alcohol and water (1 per cent H_2O in absolute alcohol). The primary H^3OH sample was standardized in a mass spectrometer and then diluted to a suitable concentration. The H^3-dihydrocholesterol also was standardized by combustion and assay of the water produced in direct comparison with a standard H^3OH sample.[11]

Because of the occurrence of quenching in some samples (e.g., 17-ketosteroid fractions), internal standards were used in counting both C^{14} and H^3. A volume of 1.00 ml. of a solution of H^3-dihydrocholesterol in toluene containing a large, known number of disintegrations per minute was added after the H^3 and C^{14} counts had been recorded. The increment in CPM per milligram of H^3 was recorded, and the relative efficiency of H^3 counting for each sample was recorded. Then 1.00 ml. of a standard solution of C^{14}-benzoic acid in toluene was added and the increment in C^{14} counting rate determined. Quenching of C^{14} counting was then calculated in the same way. No appreciable quenching of either C^{14} or H^3 was found in any of the samples reported in this paper.

TABLE I. PROTOCOL FOR DOUBLE-TRACER EXPERIMENT, CASE 1

SCHEDULE OF EVENTS, PREOPERATIVE PERIOD	
DAYS BEFORE SURGERY	EVENT
8	10 capsules H^3-cholesterol (13 μc)
7	1 capsule H^3-cholesterol (1.3 μc)
6	1 capsule H^3-cholesterol (1.3 μc) (First blood sample)
5	1 capsule H^3-cholesterol (1.3 μc)
4	1 capsule H^3-cholesterol (1.3 μc) (Second blood sample)
3	1 capsule H^3-cholesterol (1.3 μc) 40 I. U. ACTH, intramuscularly
2	1 capsule H^3-cholesterol (1.3 μc) (Third blood sample) 40 I. U. ACTH, intramuscularly
1	1 capsule H^3-cholesterol (1.3 μc) (Fourth blood sample) 40 I. U. ACTH, intramuscularly

SCHEDULE OF EVENTS, DAY OF SURGERY		
HOUR	TIME AFTER C^{14} (MINUTES)	EVENT
0700	--	1 capsule H^3-cholesterol (1.3 μc)
0758	--	20 I. U. ACTH, intramuscularly
0800	--	Preoperative medication (Fifth blood sample)
0830	--	Anesthesia started
0849	14	C_{14}-acetate, 100 μc, intravenously
0924	54	Left ovary removed
0945	75	Biopsy, tumor nodule
1000	90	Right twelfth rib removed (Sixth blood sample)
1008	98	Right adrenal removed
1019	109	Peritoneal fat biopsy
1024	114	Muscle biopsy
1024	114	Skin biopsy

ISOTOPE DOSAGES

Table I gives a typical protocol (Case 1) including the schedules of ACTH administration, blood sampling, and the removal of tissue samples, as well as the dosages of H^3- and

*We are indebted to Dr. F. N. Hayes of the Los Alamos Scientific Laboratory for both primary standards.

C^{14}-labeled compounds. The other 3 studies were similar in general, differing in the length of time that H^3-cholesterol was fed before surgery (the details are given in Table II) and in the exact time intervals elapsing between C^{14}-acetate administration and the removal of specific tissue samples (see Table III). Case 4 differed in that no malignancy was present; consequently, no ACTH was given and the adrenal glands were not removed.

TABLE II. A. DOSE OF H^3-CHOLESTEROL

DAYS BEFORE SURGERY	CASE 1 μc	CASE 2 μc	CASE 3 μc	CASE 4 μc
8	13.0	--	--	27.0
7	1.3	--	--	5.4
6	1.3	--	--	5.4
5	1.3	--	27.0	5.4
4	1.3	13.0	5.4	5.4
3	1.3	1.3	5.4	5.4
2	1.3	1.3	5.4	5.4
1	1.3	1.3	5.4	5.4
0	1.3	1.3	5.4	5.4
Total	23.4	18.2	54.0	70.2

TABLE II. B. DOSE OF 1-C^{14}-ACETATE

Cases 1 and 2 were given 100 μc of C^{14}-acetate and Cases 3 and 4 were given 200 μc on Day 0, just before surgical procedures were started, by intravenous injection.

TABLE III. TIME INTERVALS BETWEEN ADMINISTRATION OF C^{14}-ACETATE AND REMOVAL OF BIOPSY SAMPLES

TISSUES	CASE 1	CASE 2	CASE 3	CASE 4
Blood	90	104	124	90, 110
Adrenal	98	108	131	--
Liver	--	--	--	95
Skin	114	124	146	135
Ovary	19	29	R-27 L-41	R-110 L-85
Tumor	54	--	--	--
Muscle	114	115	144	130
Fat	109	109	132	--
Rib	75	89	104	--

RESULTS

1. The values for the specific activity of tritium in plasma cholesterol during the period of H^3-cholesterol feeding are graphically demonstrated for Case 1 and Case 4 in Fig. 1. When the priming dose was 10 times as large as the daily maintenance dose (Case 1), a fairly constant value was reached after 3 to 4 days of feeding. When the priming dose was 5 times the maintenance (Case 4), there was a slow decrease in specific activity with time. The equilibrium value was roughly proportional to the daily dose, not corrected for variation in body weight, efficiency of absorption, or other factors which would affect this value.

2. *Adrenal Cholesterol Synthesis.*—The C^{14}-specific activity of adrenal cholesterol, representing the relative rate of in situ synthesis, and the H^3-specific activity, representing the degree of equilibration between plasma and adrenal cholesterol, are given in Table IV.

Case 1 received H^3-cholesterol orally for 8 days before surgery. ACTH (20 units) was given intramuscularly twice a day 3 days before the operation. One and a half hours before the operation was begun, a single dose of ACTH

(20 units) was injected intramuscularly. One hundred microcuries of C^{14}-acetate was administered intravenously 98 minutes before the removal of the right adrenal and, at about the same time (90 minutes after the injection of acetate), a blood sample was taken.

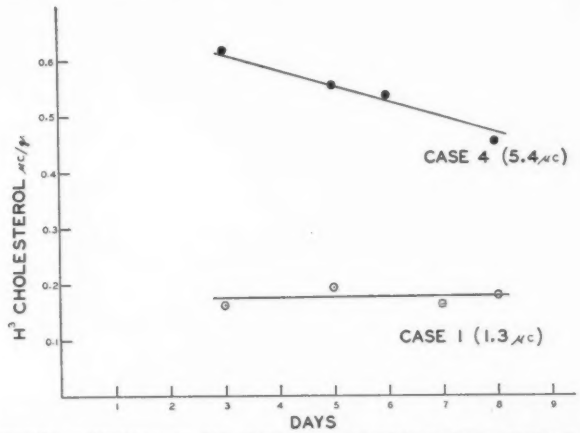

Fig. 1.—Specific activities of H^3-cholesterol resulting from feeding of the labeled sterol. In Case 1, the values shown are for whole blood total cholesterol following a priming dose 10 times the daily dose. In Case 4, the values shown are for plasma free cholesterol following a fivefold priming dose.

TABLE IV. SPECIFIC ACTIVITY OF C^{14} AND TRITIUM IN THE CHOLESTEROL OF BLOOD AND CERTAIN TISSUES
(VALUES IN μc PER 100 GM. TISSUE)

	C^{14}-SPEC. ACT. IN μC./GM. × 10^2 (CASE)				H^3-SPEC. ACT. IN μC./GM. × 10^2 (CASE)			
	1	2	3	4	1	2	3	4
Plasma, free		8.83	9.98	10.3			34.4	45.5
Plasma, ester		0.36	1.2	1.2			49.6	66.4
Red cell, free		2.0	3.6	3.1			41.5	49.1
Whole blood, total	0.46				16	12	34	--
Adrenal, free	25.2	13.9	9.0				78.4	
Adrenal, ester	4.0	2.0	1.2					
Liver				10.2				
Skin	2.55	3.8	1.0	0.68			10.6	11.1
Ovary	0.27	2.3	0.27	2.5*			19.9	27.1*
				35.7†				--†
Tumor	6.2							
Muscle	0.59		2.4	1.0			32.0	23.2
Fat	0.40	23.6	1.7				82.5	
Rib	0.90	0	2.4				69.5	

*Ovary without corpus luteum.
†Ovary with corpus luteum.

Although no plasma free cholesterol values were obtained in Case 1, the extremely low value for whole blood total cholesterol of 0.46, as compared to the adrenal free value of 25.2, indicates very strongly the occurrence of cholesterol synthesis in the adrenal. The plasma free value could not have been more than 5 times as high as the whole blood value, even if the plasma esterified and erythrocyte values had both been 0, since it constitutes about one-fifth of the whole blood total cholesterol. In all probability, it would have been about 2 to 3 times as high, judging from previous results in other patients. The mean ratio of plasma free to whole blood total specific activity values for a series

of 7 patients given 100 μc oral doses of C^{14}-acetate,[3] calculated by assuming cholesterol concentrations of 70 mg. per cent for plasma free, 140 for plasma esterified, and 140 for erythrocyte cholesterol fractions, was 2.4 at 2 hours after acetate administration. On this basis, the plasma free specific activity value in Case 1 may be taken as 1.1 and could not have been higher than 2.3, in either case far below the adrenal free cholesterol value of 25.2.

Case 2 was given H^3-cholesterol for a period of 4 days before the day of operation. ACTH was administered daily in 2 doses of 20 units each for 3 days before surgery. On the day of surgery the patient received 20 units of ACTH in the morning, and 100 μc C^{14}-acetate were given intravenously 108 minutes before the right adrenal was removed.

The C^{14}-specific activity of adrenal free cholesterol was more than 1.5 times as high as that for plasma free cholesterol, obtained from blood taken at the time the adrenal was removed. This result can only be explained by a synthesis of cholesterol in the adrenal itself, since many studies on other patients have shown that the plasma free cholesterol specific activity value does not reach a maximum until about 3 hours after the administration of C^{14}-acetate. At 108 minutes it was rising rapidly and the adrenal could have reached a higher value only by independent synthesis of cholesterol.

In Case 3, after a 5-day period of H^3-cholesterol feeding and after a 3-day period of ACTH administration (20 units 2 times daily), 20 units of ACTH and 200 μc of C^{14}-acetate were given on the morning of the operation. The right adrenal was removed 131 minutes after the administration of the acetate and a blood sample was taken 7 minutes before the excision of the adrenal. Although the plasma free cholesterol C^{14}-specific activity value was somewhat higher than that of adrenal free cholesterol, the short time during which a high plasma value could have existed would not have sufficed to permit much equilibration. This result is certainly in accord with adrenal cholesterol synthesis, although by itself it is inconclusive.

In Case 4, neither adrenal was removed.

3. *Liver.*—In Case 4, a liver biopsy was obtained and the C^{14}-specific activity found to be approximately the same for plasma free as for liver cholesterol. This liver biopsy was taken at 95 minutes and the blood sample at 90 minutes after the administration of C^{14}-acetate. The equality at this time confirms our previous assumption[1] that the time relationships of unesterified cholesterol molecules in liver and in plasma are similar to those in dogs, except that the peak values are reached more slowly in humans.

4. *Skin.*—The C^{14}-specific activity values of skin cholesterol were significant (about double the background in both Geiger-Mueller end window and liquid scintillation counters) but they were considerably lower than the plasma free values. Since skin cholesterol has been found to equilibrate very slowly with plasma (not more than 30 per cent equilibration in Cases 3 and 4 after 5 and 8 days, respectively, of exposure to high plasma H^3-cholesterol specific activities), it is reasonable to assume that most of the C^{14}-cholesterol isolated from the skin biopsies has been synthesized in situ. This conclusion is confirmed by the results of an in vivo incubation of a skin slice. A 480 mg. sample of normal

human skin (obtained by means of a dermatome) was incubated in Krebs' bicarbonate buffer solution with 7.70 μM of 1-C^{14}-acetate for 3 hours at 37° C. The cholesterol isolated from the skin as digitonide had 181 counts per minute. This represents 0.11 per cent of the C^{14} in the acetate and corresponds to a turnover rate in the order of magnitude of 0.01 per cent per hour.

5. *Tumor.*—The single value obtained on a biopsy of a mammary carcinoma metastasis was of interest because the value for C^{14} found suggested that this neoplastic tissue was capable of cholesterol synthesis.

6. *Ovary.*—The C^{14} values for the first 3 patients were so low as to suggest negligible synthesis. In these patients the ovaries were removed early (from 27 to 41 minutes after acetate administration), which was probably before the completion of cholesterol synthesis. More significant is the fact that the ovaries were nonfunctional in these patients either because of age or previous treatment. Consequently, this result is not unexpected. In Case 4, however, the ovary containing a corpus luteum was extremely active; the other ovary was also active, although much less so. In a series of patients studied more recently at the Chicago Lying-in Hospital in collaboration with Drs. Davis and Plotz, it has been found that the corpus luteum itself has a very rapid rate of cholesterol synthesis in vivo. These results have been reported in abstract form[12] and will be described in detail elsewhere.

7. *Other tissues.*—The high value for depot fat cholesterol in Case 2 was not in agreement with the same tissue in other patients and must be regarded as an aberrant result. This sample may have been contaminated with adrenal ectopic tissue or a tumor metastasis. The evidence in these cases does not establish cholesterol synthesis in human adipose tissues.

Specific activity values of C^{14}-cholesterol in muscle and rib are lower than the corresponding plasma free cholesterol values and may be due to a slow rate of synthesis, to a rapid equilibration with plasma cholesterol, or to both.

8. *Rate of Equilibration of Plasma and Tissue Cholesterol.*—The data on H^3-cholesterol specific activities obtained in the first 2 cases studied by the double-labeling technique were unsatisfactory and are not shown in Table IV. This was due in part to the low doses (1.3 μc per day) of tritium-cholesterol used, and in part to the fact that the technique for counting tritium and radiocarbon simultaneously was in the developmental stage when the first 2 studies were done. When a larger dose (5.4 μc per day) of tritium-cholesterol was used, as in Cases 3 and 4, the data are considered to be reliable. Table V gives the actual counting rates for the specific activity data for Case 4. As a result of experience with the patients described in this report, the dose of tritium-cholesterol used in subsequent studies has been increased to approximately 10 μc per day, an amount which provides an adequate number of tritium counts in the presence of the usual values for C^{14}-cholesterol.

The extent to which equilibration of plasma and tissue H^3-cholesterol occurs can be inferred from comparisons of specific activity. The present study obviously does not permit the assignment of a value for this rate in any tissue, but the results given in Table IV are suggestive.

TABLE V. OBSERVED COUNTING RATES IN CASE 4

SAMPLE	MILLIGRAMS COUNTED	CPM AT C^{14} SETTING = C^{14} CPM	CPM AT H^3 SETTING	CPM FOR H^3 CORR. FOR C^{14}
90-Minute Sample				
Plasma free cholesterol	2.11	127	203	135
Plasma esterified cholesterol	3.36	25	330	318
Erythrocyte cholesterol	3.65	66	287	252
110-Minute Sample				
Plasma free cholesterol	1.99	56	115	85
Plasma esterified cholesterol	2.69	32	566	549
Erythrocyte cholesterol	3.65	76	308	270
Left ovary free cholesterol	2.25	460	258	2 (?)
Left ovary esterified cholesterol	5.08	195	206	102
Right ovary free cholesterol	2.69	39	126	104
Right ovary esterified cholesterol	5.35	10	87	82
Skin	2.28	9	42	37
Muscle	2.30	13	85	78

DISCUSSION

The results of our studies show that nondiseased adrenal glands are able to utilize C^{14}-acetate for the synthesis of cholesterol in vivo. It should be noted, however, that these adrenal glands were maximally stimulated by ACTH. Recently, Smith and Werthessen,[15] using the in vitro technique of isolated organ perfusion, demonstrated the utilization of 1-C^{14}-acetate as substrate for the cholesterol synthesis in a human adrenal tumor. Previously, cholesterol synthesis in the adrenals of animals has been reported by various groups of investigators using different techniques. Srere, Chaikoff, Friedman, and Burstein[16] found that beef adrenal cortex slices are capable of the transformation of labeled acetate to cholesterol. The perfusion of beef adrenals by Hechter, Solomon, Zaffaroni, and Pincus[17] resulted in the conversion of added C^{14}-acetate to cholesterol.

The H^3-cholesterol specific activity values in the various tissues biopsied demonstrates that dietary cholesterol appears not only in blood but also in many tissues in humans. The results for individual tissues show varying degrees of equilibration and indicate that the concept of a single pool of metabolically active cholesterol is untenable, in agreement with conclusions previously reached from studies on experimental animals.[14]

In a series of dogs injected intravenously with C^{14}-cholesterol in the form of normal dog lipoproteins (prepared by administration of C^{14}-acetate to donor dogs), it was shown that each tissue has its own individual rate of equilibration of cholesterol between plasma and tissue cell. However, it is possible that an apparent pool size, empirically derived by measurement of the dilution of a small amount of labeled cholesterol at a given time, may be useful clinically in detecting abnormalities in cholesterol metabolism. Labeled cholesterol may be introduced in several different ways: (1) the intravenous injection of blood obtained from a donor given labeled cholesterol, or a labeled precursor of cholesterol, as was done in the dog studies previously referred to[14]; (2) the injection of labeled cholesterol in emuslified form; and (3) the feeding of labeled cholesterol as in the present studies. The third method is simple and free from certain drawbacks of the first 2 methods, but has the disadvantage that oral

absorption of cholesterol is never complete. The absorption of large doses of cholesterol (e.g., 0.7 to 3 Gm.) has been found to be in the range of 34 to 77 per cent complete.[18-20] Although the efficiency of absorption has not been determined for doses as low as those in the present study, it is well known that the absorption decreases in completeness as the dose is increased, so that a reasonable estimate of the absorption in these patients is approximately 75 per cent. Using this figure, an "apparent" pool size has been estimated for the 4 patients studied and the results are given in Table VI. The mean value is 61 Gm., in good agreement with values obtained by the direct injection of C^{14}-cholesterol (after allowing 7 days for equilibration) both by Kurland, Lucas, and Freedberg[21] who found 26 to 55 Gm., and by LeRoy and associates,[22] who found 27 to 34 Gm.

TABLE VI. ESTIMATES OF APPARENT CHOLESTEROL POOL SIZE AND APPARENT TURNOVER RATE

CASE NUMBER	APPARENT POOL SIZE (THIRD DAY)	APPARENT TURNOVER RATE (GM./DAY)
1	57	5.7
2	78	7.8
3	58	11.6
4	50*	9.9
mean	61	8.8

*This value is based on the specific activity value on Day 3. The pool size would be 68 Gm. if calculated from the specific activity value on Day 8. The apparent pool size values were calculated from the equation: μc H^3—cholesterol absorbed = μc/mg. blood cholesterol × mg. in apparent pool.

Only the labeled cholesterol administered in the priming doses was taken into account in this calculation, since the blood specific activity values show that the later doses merely seemed to replace the amount of labeled cholesterol which left this "apparent" pool. This observation makes it possible to estimate the daily turnover of labeled cholesterol in the "apparent" pool. If a daily dose of 5.4 μc is absorbed to the extent of 75 per cent and does not increase the specific activity of the cholesterol in this "apparent" pool, then an equal number of microcuries of pool cholesterol must have left the pool. Amounts of cholesterol corresponding to this number of microcuries may be estimated from the blood cholesterol specific activity values and are given in Table VI.

The "apparent" pool is obviously much larger than the cholesterol of blood (ca. 6 Gm.), liver (ca. 5.4 Gm.), and spleen (ca. 0.4 Gm.). It must include most, if not all, of that in other viscera and at least a portion of that in skeletal muscle, adipose tissue, and, perhaps, skin. Studies to determine equilibration rates of cholesterol between plasma and a wide variety of tissues, including arteries, have been made more recently on terminal patients and will be reported elsewhere.[23]

SUMMARY

1. A method is described for estimation of extrahepatic synthesis of cholesterol in human subjects by the analysis of tissue biopsy samples a short time after the administration of C^{14}-acetate.

2. Equilibration of cholesterol between plasma and various tissues may be estimated simultaneously by administering tritium-labeled cholesterol for an

appropriate period before the surgical procedure. The specific activity values for both C^{14} and H^3 in the same sample were determined by counting in a liquid scintillation counter at 2 settings.

3. Adrenal gland stimulated by ACTH and the ovary containing an active corpus luteum were shown to synthesize cholesterol rapidly from acetate. Definite, although much slower, rates of cholesterol synthesis were observed in skin which were confirmed by an in vitro study on human skin. Other tissues examined failed to give convincing evidence of extrahepatic synthesis.

4. All tissues showed the presence of H^3-cholesterol which had been derived from that administered orally. Differences between the specific activity in plasma and in the tissues studied support the concept that rates of equilibration vary widely with different tissues.

5. These experiments demonstrate the feasibility of a double isotopic labeling technique for investigation of the metabolism of cholesterol in man.

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THE EFFECTS OF HYPOXIA UPON THE LIPEMIA-CLEARING RESPONSE TO INTRAVENOUS HEPARIN

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INTRODUCTION

RECENT work has indicated that clearing-factor, formed following the intravenous injection of heparin, is a lipolytic enzyme which is capable of clearing the turbidity of lipemic serum in vivo or in vitro.[1] A physiologic role of clearing-factor in normal lipid metabolism is assumed[1, 2] and its relationship to various diseases of lipid metabolism has been reported.[3-6] Endogenous clearing-factor has been found in the plasma of animals following anaphylactic shock[7-9] and also in selected normal humans.[10]

It was previously reported from this laboratory that hypoxia inhibited the formation and action of heparin-induced clearing-factor.[11] In view of the possible importance of oxidative mechanisms in the development of atherosclerosis and in lipid metabolism these experiments are reported in full.

MATERIALS AND METHODS

Dogs weighing between 7 and 14 kilograms were used in these experiments. An alimentary lipemia was produced by the feeding of 50 Gm. of olive oil and allowing 4 to 6 hours for suitable lipemia to develop following the fatty meal. All animals were anesthetized (as lightly as possible) by small doses of Nembutal (approximately 30 to 35 mg. per kilogram). All blood samples obtained during the experiments were drawn into sodium citrate and centrifuged within 5 minutes. The degree of lipemia in plasma samples was determined by measuring the per cent transmittance of a constant light source through the sample. This was done in a Beckman Model B Spectrophotometer using a blue-sensitive photoelectric tube and a wave length of 550 mμ. The spectrophotometer was balanced against a saline blank at 100 per cent transmittance and the plasma samples were diluted one part plasma in nine parts normal saline before reading. Thus, the greater the per cent transmittance, the less the optical density and the greater the clearing presumed to have occurred. Samples were diluted and read immediately after centrifugation, after which the diluted samples were incubated at 37° C. and followed for in vitro clearing over a 6- to 18-hour period.

In general, the pattern of clearing was determined from samples drawn before heparin, 5 minutes, 10 minutes, and, when possible, 15 to 30 minutes after heparin. The usual dose of heparin (Abbott Sodium Heparin) was 30 mg. intravenously. The per cent of maximum clearing possible for any given sample is determined by the formula

$$\frac{R_2-R_1}{98-R_1}\times 100$$

where R_1 is the control reading (in per cent transmittance) of the plasma sample before

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TABLE I. THE PER CENT MAXIMUM CLEARING POSSIBLE OF LIPEMIC OPTICAL DENSITY FOLLOWING 30 MG. HEPARIN INTRAVENOUSLY IN 4 DOGS SUBJECTED TO ANOXIC HYPOXIA BY REBREATHING

Rebreathed Dogs	5 minutes after Heparin	10 minutes after Heparin	15 minutes after Heparin	Duration IN VITRO Clearing	Increased Plasma O.D. after Rebreathing
DOG 1-R	1% / 26%	3% / 33%	3% / 40%	7.5 HRS.	+
DOG 2-R	16% / 56%	25% / 75%	58% / 37%	9 HRS.	+
DOG 3-R	14% / 55%		17% / 35%	2 HRS.	+
DOG 4-R	35% / 45%		81% / 38%	0.5 HRS.	−

In vivo changes (see text) are represented above and to the left of the diagonals. In vitro changes (see text) are represented below and to the right of the diagonals. Note that the plasma of 3 animals became more turbid after rebreathing and before the injection of heparin.

heparin, and R_2 is the reading of any given sample following heparin. The figure 98 represents the maximum per cent transmission to which clearing can occur, since that is the highest value obtained on the spectrophotometer by a 1:9 dilution of clear plasma in saline. The same formula is used to determine the per cent maximum clearing possible in in vitro samples. Here R_1 is the initial reading of any given plasma sample and R_2 is the reading of the same sample following some period of incubation.

The in vivo lipemia clearing effect of heparin in the normal dog made lipemic by olive oil has been described in a paper from this laboratory.[12] It was observed that approximately 77 per cent of the total amount of clearing possible occurred within 5 minutes; i.e., 77 per cent maximum clearing possible. This 77 per cent value is considered the approximate normal in the experiments to be presented and inhibition of clearing is assumed to have occurred when no greater than 60 per cent of maximum clearing possible has occurred 5 minutes following heparin.

In normal lipemic dogs, the plasma optical density following heparin is relatively clear in 5 minutes. Therefore, an estimation of the clearing-factor content as evidenced by subsequent in vitro clearing cannot be made by rereading the optical density of the incubated samples. For this reason the following experiments were done. Six dogs were made lipemic by being fed olive oil and plasma samples were obtained to be used as the clearing substrate for samples withdrawn 5, 10, and 30 minutes following heparin injection. The preheparin lipemic plasma and the postheparin "clearing" plasma were mixed in equal parts and diluted 1:9 with normal saline. Postheparin samples were incubated at 37° C. for 8 hours with the lipemic plasma substrate obtained from the same animal. Control values were obtained by incubating the lipemic substrate in a 1:9 dilution of normal saline. The mean values for in vitro clearing in these experiments for plasma samples obtained 5, 10, and 30 minutes

postheparin were, respectively, 78, 79, and 80 per cent of maximum clearing possible. The mean values for in vitro clearing of the preheparin lipemic substrate were 14 per cent of maximum clearing possible.

In experiments where an inhibition of heparin-induced lipemia clearing was produced, the plasma was sufficiently lipemic to allow a study of subsequent in vitro clearing. It was considered that the addition of lipemic substrate to these samples would obscure the results. Arbitrarily, values less than 60 per cent maximum clearing possible were considered inhibited in these in vitro experiments.

RESULTS

1. *Experiments Demonstrating the Alteration in the Lipemia-Clearing Effect of Heparin After Rebreathing.*—Anoxic hypoxia was produced in 4 dogs by allowing each animal to rebreathe into a Blalock dog mask for at least 5 minutes before and 5 minutes following heparin. One dog was made to rebreathe for 10 minutes following heparin. Plasma samples were obtained before rebreathing, just prior to the injection of heparin, and 5, 10, and 15 minutes following the injection of heparin. These samples were studied immediately for in vivo clearing and subsequently followed for in vitro clearing.

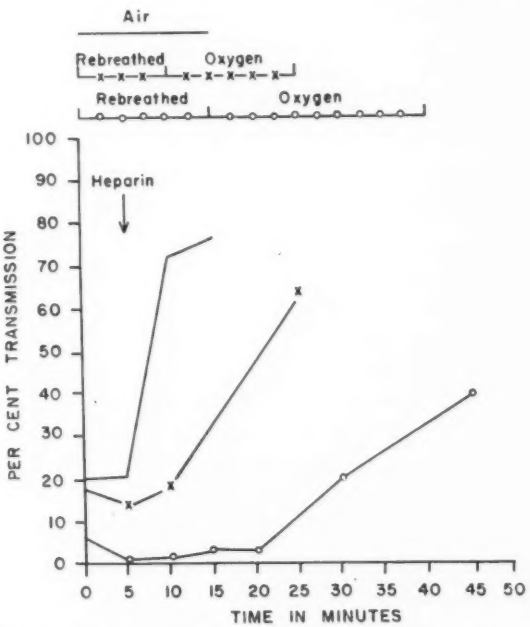

Fig. 1.—The per cent changes in lipemic optical density following 30 mg. of intravenous heparin in a normal dog and in 2 dogs subjected to anoxic hypoxia by rebreathing. Values were recorded immediately after centrifugation of samples. Note that the longer the animal was rebreathed, the greater and longer clearing was inhibited.

Fig. 1 presents the typical clearing pattern of a normal dog as contrasted to that of 2 hypoxic dogs in this experiment. Table I includes the results of these experiments in tabular form with in vivo clearing represented above and to the left of the diagonal and in vitro clearing below and to the right of the diagonal. It can be seen that significant inhibition of in vivo clearing occurred in all animals. It should be noted that, though less than normal, in vitro clearing was evident in each of the above samples when incubated at 37° C.

2. *The Modification of the Lipemia-Clearing Effect of Heparin Following Sodium Cyanide.*—Seven dogs were used in experiments demonstrating the effects of cyanide upon lipemia clearing by heparin. Six animals received 3 to 12 mg. of a 0.1 per cent solution of sodium cyanide in physiologic saline intravenously from 4 to 8 minutes before heparin. One animal received 10 mg. sodium cyanide one-half hour before heparin. In all but 2 dogs a plasma sample was drawn just before heparin, and samples were drawn in all animals at 5, 10, and 30 minutes following heparin. In vitro clearing of samples incubated at 37° C. was followed in each experiment for 16 hours.

In all animals from which samples were drawn after cyanide, but before heparin, there was an increase in plasma optical density. This response is accompanied by an increase in the number of chylomicrons as observed by phase microscopy and has been observed following rebreathing and the injection of protamine sulfate into normal lipemic dogs. The increase in turbidity may be due in part to the inhibition of endogenous clearing-factor which is assumed to be present.[10] Lipemia clearing by heparin in the dogs injected with cyanide was markedly inhibited 5 minutes after heparin in 6 animals, and even reversed in 4 of these 6. In the animal receiving cyanide one-half hour before heparin (Dog 7-C), clearing was inhibited to a lesser degree than in the other animals. Five of the 7 animals showed considerable inhibition of clearing 30 minutes after heparin. In vitro clearing was inhibited in 4 animals, but it occurred in the remaining 3 to about the same degree as in the rebreathing experiments. Table II shows the per cent of the maximum clearing possible that had occurred in vivo at 5, 10, and 30 minutes postheparin in each of the 7 animals, and also the per cent of the maximum clearing possible that had occurred in vitro after the samples had been incubated for 16 hours.

3. *The Effects of 2, 4-Dinitrophenol on Lipemia Clearing by Heparin.*—To determine if oxidative phosphorylation was involved in the formation and action of heparin-induced lipemia clearing-factor, 2, 4-dinitrophenol in a saturated solution with the pH adjusted to 7.4 was injected intravenously into 13 dogs made lipemic by being fed olive oil. Fifteen to 60 minutes later, heparin was injected and plasma samples obtained and followed for in vivo and in vitro clearing as described above. The complicating factors of hyperthermia and terminal hypoxia made much of the data inconclusive; however, in the animals where these factors were minimal, no inhibition of in vivo or in vitro clearing was observed.

4. *In Vitro Observations: Factors Which Inhibit and Facilitate Clearing.*—Two other methods of determining in vitro clearing were employed in addition to that described in the above experiments; however, these methods differ only by virtue of the turbid medium in which clearing ability was tested. One of these media consisted of a 1:8 dilution of lipemic normal dog plasma in saline, while the other was a 0.25 per cent solution of a special fat emulsion* in a phosphate buffer (pH-8). These media were used to test factors which might

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inhibit or facilitate the in vitro clearing ability of plasma samples from heparinized normal dogs, as well as the clearing ability of clearing-factor prepared in vitro by incubating heparin and dog plasma with an homogenate of fresh dog pylorus. The in vitro clearing-factor was prepared by techniques described by Anfinsen, Boyle, and Brown.[2] Clearing was observed over a 6-hour period.

TABLE II. THE PER CENT MAXIMUM CLEARING POSSIBLE OF LIPEMIC OPTICAL DENSITY FOLLOWING 30 MG. HEPARIN INTRAVENOUSLY IN 7 DOGS SUBJECTED TO CYTOTOXIC HYPOXIA BY SODIUM CYANIDE

Cyanide Dogs	5 minutes after Heparin	10 minutes after Heparin	30 minutes after Heparin
DOG 1-C	1% / 7%	3% / 19%	20% / 0%
DOG 2-C	0% / 4%	0% / 3%	18% / 55%
DOG 3-C	-13% / 75%	-23% / 56%	76% / 0%
DOG 4-C	-2% / 7%	-2% / 5%	2% / 17%
DOG 5-C	7% / 70%	8% / 76%	28% / 0%
DOG 6-C	-10% / 38%	26% / 39%	8% / 13%
DOG 7-C	26% / 95%	70% / 93%	70% / 46%

Minus values represent increased optical density. In vivo changes are represented above and to the left of the diagonals. In vitro changes are represented below and to the right of the diagonals and were read over a 16-hour period of incubation.

Inhibition of in vitro clearing was tried with sodium cyanide, 2, 4-dinitrophenol (DNP), constant perfusing of the system with CO_2, and maintenance of a system which was as anaerobic as possible. The latter was done by keeping the entire clearing system under oil or by constant perfusion of the incubating system with nitrogen or illuminating gas. Facilitation was attempted by constant perfusion of the clearing system with air or oxygen.

From the in vitro experiments using both the fat emulsion and lipemic dog plasma as substrates, the following observations were made: (a) Sodium cyanide added in vitro did not inhibit in vitro clearing. (b) DNP added in vitro did not inhibit in vitro clearing. (c) In vitro clearing was not inhibited when incubated under oil or when constantly perfused with nitrogen or illuminating gas. (d) Constant perfusion of the incubating clearing system with CO_2 inhibited clearing, but also caused a marked pH change even in the buffered system. (e) Constant perfusion of the incubating clearing system with air, oxygen, nitrogen, or illuminating gas markedly facilitated clearing in the fat emulsion media, while to a less marked and less consistent degree in the lipemic plasma media.

DISCUSSION

The data in this report show that the normal lipemic dog's clearing response to intravenous heparin can be consistently modified by rebreathing or by sodium cyanide. Since both rebreathing and cyanide have in common the production of a form of hypoxia, it seems reasonable to assume that hypoxia plays a definite role in the inhibition of lipemia clearing following heparin.

It is of interest in this respect that animals in which anemia is produced by repeated hemorrhages or hemolytic agents develop a lipemia.[13-15] Injections of red blood cells into these animals abolished the lipemia and reduced the blood lipids to normal, while serum did not.[15] Recently, Spitzer and Spitzer[16] have reported that rabbits with hemorrhagic lipemia have an impaired ability to form clearing-factor following heparin, but, though anemic, did form it before the lipemia developed and during the recovery phase. Starup[15] reported that rabbits subjected to reduced atmospheric pressure with less than half normal partial pressure of oxygen became lipemic, and he concluded that this lipemia as well as hemorrhagic lipemia was due to lack of oxygen.

The effect of increased plasma CO_2 tension and the concomitant fall in plasma pH that attends prolonged rebreathing are not considered important factors influencing the lipemia clearing in these experiments since the range of efficient clearing-factor activity in in vitro studies is wide enough to exclude the pH changes produced in vivo,[17] and similar results were obtained in two experiments where CO_2 was removed by soda-lime. In vitro studies showed a definite inhibition of clearing when CO_2 was bubbled through an incubating clearing system. However, the pH changes during this procedure could account for the inhibition.

Cyanide, in contrast to rebreathing, produces cytoxic hypoxia with an actual increase in oxygenation of the venous blood,[18] yet is a more effective inhibitor than rebreathing. This inhibition is presumed to be due to hypoxia at the cellular level as the result of interference with aerobic oxidation through the cytochrome oxidase system.[19] The fact that cyanide poisoning was followed in three instances by less than 10 per cent maximum possible in vitro clearing suggests that there must have been an actual inhibition of clearing-factor formation, especially since cyanide added in vitro causes no inhibition of in vitro clearing.

The lipemia clearing following hypoxia produced by rebreathing was inhibited in vivo but was relatively efficient in vitro. This would suggest that some clearing-factor was formed but unable to act in vivo. In vitro experiments have demonstrated that clearing-factor formed in vivo cannot be inhibited by incubation under oil or by perfusion with nitrogen or illuminating gas.

The rate of clearing is in no way comparable in the two systems, clearing in vivo being much more rapid than in vitro. Thus, hypoxia actually may convert the in vivo rate of clearing to an essentially in vitro one by inhibiting whatever processes are responsible for the great temporal difference between the two, such that already formed clearing-factor acts in vivo at a typical in vitro rate. If this be true, then one would expect the 5-minute sample following heparin to keep pace with in vivo clearing in the hypoxic animal from which the sample was obtained. This is what happened in one animal over a 30-minute period. However, even that animal could not be maintained at the initial degree of hypoxia for such a period of time, and no subsequent animal could be so maintained for even a third this long.

Supplying oxygen to the rebreathed animals will effect an increase in clearing. This, of course, may allow increased formation of clearing-factor rather than allowing that which is already formed to act. When air or oxygen was perfused through the in vitro incubating samples a definite facilitation of clearing occurred. However, it also occurred when perfused with nitrogen or illuminating gas. It appears, therefore, that the agitation was responsible for the increased clearing rate rather than the oxygenation, probably by providing more adequate access of the "lipemic substrate" to clearing-factor. Although such perfusion with gas is less effective and even inconsistent when lipemic plasma is used instead of the artificial "clearing media," it has become useful as an expedient technique, particularly when using this artificial menstruum to test clearing-factor prepared in vitro. This enhancement by agitation may be a small factor in the rate difference between the constantly agitated in vivo system and the usually stagnant in vitro system.

Thus it would appear that both the in vivo formation and the in vivo action of clearing-factor are dependent upon an aerobic phase of metabolism; that in vitro, clearing-factor action is facultatively anaerobic; that in vivo clearing-factor formation and in vivo clearing-factor action are apparently dependent upon two separate dynamic mechanisms; that in vivo clearing-factor formation is susceptible to both anoxic hypoxia and cytotoxic hypoxia and varies proportionately with the degree of hypoxia produced; and finally, that in vivo clearing-factor action is susceptible to anoxic hypoxia, probably being reduced in rate to that seen in vitro.

SUMMARY

1. The usual clearing response of a lipemic animal to heparin was significantly inhibited by rebreathing.

2. Intravenous sodium cyanide inhibited lipemia clearing following heparin to an even greater degree than rebreathing. Results with 2, 4-dinitrophenol were

indefinite; however, it is probable that this chemical did not significantly inhibit clearing.

3. Attempts at inhibiting and facilitating in vitro clearing are reported.

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THE RESPONSIBILITY OF SPERMINE FOR THE ANTIBACTERIAL ACTION OF HUMAN SEMEN

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THE growth-inhibiting effect of human semen on various bacteria was described by Rozansky and co-workers[1] and by Taylor and Morgan.[2] Gurevitch and associates[3] reported on the antibacterial action of spermine, which is a normal constituent of human semen. They observed a similarity between the growth-inhibiting effect of human semen and spermine and suggested that the latter is the factor responsible for the antibacterial effect of human semen. This report describes experiments which provide additional evidence for this suggestion.

The experiments were based on the examination of the antibacterial action of semen from which spermine had been eliminated by preincubation with sheep serum which is known to contain spermine-oxidase.[4] The action of human semen on a spermine resistant strain of *Staphylococcus aureus* also was investigated.

MATERIALS AND METHODS

Semen.—Samples of semen were collected from patients at the sterility clinic. The samples were allowed to liquefy and were then centrifuged. The supernatant plasma was used either immediately or after storage at -10° C.

Sheep Serum.—Blood was taken from the jugular vein of sheep and defibrinated with glass beads. The serum was sterilized by Seitz filtration and used immediately or kept at 4° C. for a period not exceeding one week.

Spermine tetrahydrochloride* was used throughout the experiments.

Test Organisms.—Staphylococcus aureus No. 23, sensitive to 12 mcg./ml. of spermine and a mutant of this strain resistant to 4,000 mcg./ml. of spermine served as test organisms. The resistant strain was obtained by subculturing the sensitive one through increasing concentrations of spermine in nutrient broth (Difco) at a pH 7.4.

Antibacterial Tests.—0.2 ml. of a 10^{-3} dilution of a 24-hour-old broth culture of the test strains was dispersed on the surface of Difco nutrient agar plates (pH 7.4) by a Drigalski rod. Sterile porcelain cups, 8 mm. in diameter (external), were placed on the inoculated plates. Each cup received 3 drops of the solution to be tested. The results were read after 24-hour incubation at 37° C.

Analytical Methods.—O_2 uptake was measured by the conventional Warburg manometric technique.[5] Ammonia was determined by distillation in a Markham still.[6] The quantitative determination of spermine was performed by paper chromatography. Whatman No. 1 paper was used. The developing solvent was butanol:acetic acid:water, in the

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ratio 2:1:1. Time of run was 24 hours at room temperature. The paper was air dried and sprayed with 0.5 per cent ninhydrin solution in butanol. The color then was developed by placing the chromatogram in an oven at 60° C. for 30 minutes. The R_F value of spermine was 0.11. The colored spots were cut off and extracted with 4 ml. of a 75 per cent ethanol solution containing 0.2 mg. of $CuSO_4 \cdot 5H_2O$.[7] The color intensity was determined by a Klett-Summerson photoelectric colorimeter using a 540 mμ filter and compared to a standard curve.

All experiments were done in triplicate.

RESULTS

Enzymatic Degradation of Spermine in Semen by Sheep Serum.—Oxygen was taken up and ammonia set free when semen and spermine were incubated with sheep serum. Fig. 1 shows that the initial rate of oxidation was identical with semen and spermine.

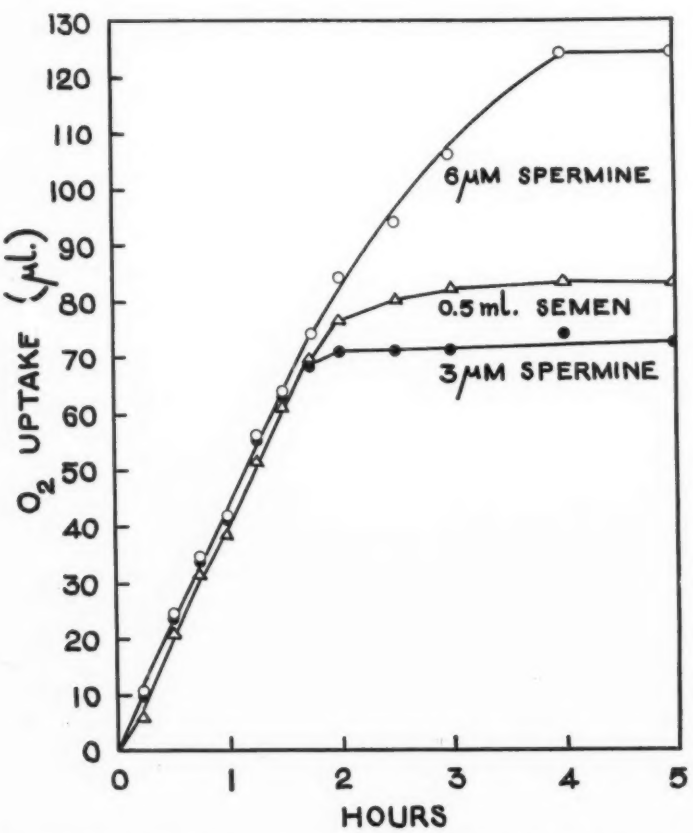


Fig. 1.—O_2 uptake of semen and spermine incubated with sheep serum. Each Warburg vessel contained 0.5 ml. of substrate, 0.6 ml. of sheep serum, and 1.9 ml. of M/15 phosphate buffer, pH 7.0. 0.2 ml. of 15 per cent KOH was in the center well. Temperature, 30° C. Atmosphere, air. The values have been corrected for endogenous activity of both semen and sheep serum.

It may be seen from Table I that when 3 μM of spermine was incubated with sheep serum, 1 μM of O_2 was taken up and 2 μM of NH_3 liberated for each molecule of spermine oxidized. These figures show that all of the spermine

present was oxidized.[8] However, when 6 μM of spermine was used, the oxygen uptake and ammonia production were less than expected, indicating incomplete oxidation of spermine.

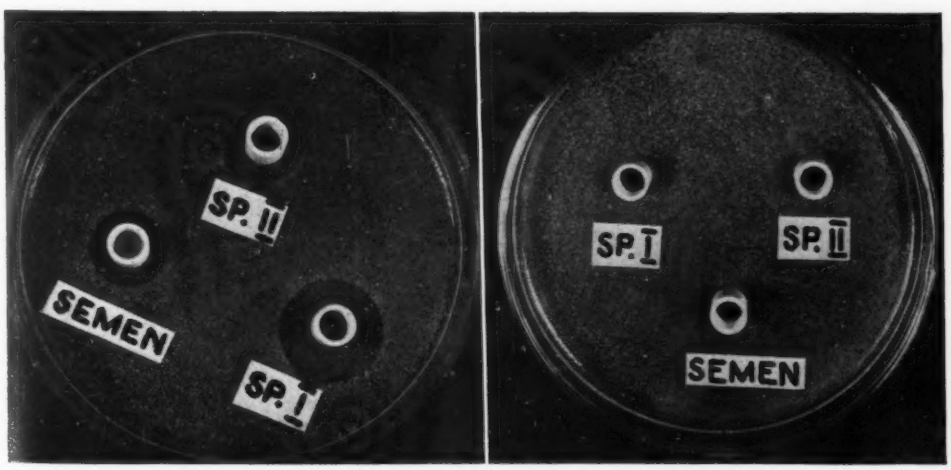
The ratio of O_2 uptake per NH_3 liberated when semen served as substrate was the same as the ratio found with spermine as substrate (Table I). These findings indicate that spermine was the substance oxidized in semen.

TABLE I. OXYGEN UPTAKE AND AMMONIA PRODUCTION OF SEMEN AND SPERMINE DURING OXIDATION BY SHEEP SERUM SPERMINE-OXIDASE*

SUBSTRATE	OXYGEN UPTAKE (μM O_2)	AMMONIA PRODUCTION (μM)	O_2 UPTAKE PER AMMONIA LIBERATED
3 μM spermine	3.2	5.7	0.56
6 μM spermine	5.5	9.9	0.55
0.5 ml. semen	3.6	6.6	0.54

*Flask contents as in Fig. 1. Time of incubation, 5 hours.

Diminution of the Antibacterial Action of Semen Treated With Sheep Serum.—Four parts of semen and one part of sheep serum were incubated in a water bath at 37° C. At various time intervals as specified in Table II, samples were taken and heated at 70° C. for one hour to inactivate the spermine-oxidase. The heated samples were tested for their antibacterial activity by the cup method. *Staphylococcus aureus* No. 23 served as test organism. A similar experiment with a spermine solution (800 mcg./ml.) was done simultaneously (Table II). The antibacterial effect of both semen and spermine decreased with increasing incubation time in the presence of sheep serum. The presence of a small amount of unoxidized spermine could be demonstrated chromatographically following the treatment of spermine with sheep serum even after incubation for 5 hours. Spermine could not be demonstrated chromatographically in semen because of interfering substances.



A. *B.*

Fig. 2.—Influence of semen and spermine on the growth of *Staphylococcus aureus*. *A*, *Staphylococcus aureus* No. 23. *B*, The spermine-resistant mutant. Sp.I. = 4,000 mcg./ml. spermine. Sp.II = 400 mcg./ml. spermine.

TABLE II. ANTIBACTERIAL ACTION OF SEMEN AND SPERMINE AFTER PREINCUBATION WITH SHEEP SERUM

TIME OF PREINCUBATION (MINUTES)	SEMEN	SPERMINE TETRAHYDROCHLORIDE	
	INHIBITION ZONE (DIAMETER IN MILLIMETERS)	INHIBITION ZONE (DIAMETER IN MILLIMETERS)	RESIDUAL SPERMINE (MCG./ML.) (DETERMINED CHROMATOGRAPHICALLY)
0	17.5	14	800
15	16.5	13	600
60	15.0	11	400
180	—	10	200
240	12.5	—	—
300	8.0	9	160

Experiments With the Spermine-Resistant Strain.—In experiments with *Staphylococcus aureus* No. 23, an inhibition zone of 15 mm. in diameter was observed around the semen containing cup, and zones of 20 and 15 mm. were measured around the cups containing a spermine solution of 4,000 and 400 meg./ml., respectively. The spermine-resistant strain was inhibited neither by semen nor by the above-mentioned concentrations of spermine (Fig. 2).

DISCUSSION

The lysozyme-like substance present in semen[2] did not affect the growth of our test strain. No constituents of human semen other than spermine and spermidine are known to exhibit an antibacterial effect at concentrations in which they occur in semen. Degradation of spermine by sheep serum spermine-oxidase was accompanied by parallel decrease in its growth-inhibiting activity.

Incubation of semen with sheep serum was accompanied by O_2 uptake and ammonia production in a ratio indicative of spermine oxidation and resulted in a decrease in the antibacterial action of the semen. In the case of both spermine and semen, the observed residual antibacterial activity following preincubation with sheep serum may be explained by the presence of a small amount of unoxidized spermine.

All of the results obtained with spermine are applicable to spermidine. Spermine and spermidine possess similar antibacterial properties[9] and *Staphylococcus aureus* was found to develop cross resistance toward both substances. Thus, the strain resistant to 4,000 meg./ml. of spermine was also resistant toward 10,000 meg./ml. of spermidine. Both substances are oxidized by spermine-oxidase.[4] The antibacterial action of semen is due mainly to spermine as spermidine is present in a much lower concentration.

SUMMARY AND CONCLUSIONS

1. Human semen and spermine inhibited the growth of *Staphylococcus aureus.*

2. Semen and spermine showed a pronounced decrease in their antibacterial action after enzymatic degradation by spermine-oxidase.

3. A spermine-resistant mutant obtained from the sensitive strain was not inhibited by human semen.

4. It is concluded that spermine and spermidine are the factors responsible for the antibacterial effect of human semen.

The authors wish to express their indebtedness to Prof. A. Brzezinsky, head of the sterility clinic, for valuable help.

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INQUIRY INTO CAUSES OF LOWERED SPINAL FLUID SUGAR CONTENT: IN VIVO AND IN VITRO OBSERVATIONS

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LOWERED cerebrospinal fluid sugar is seen in patients with acute purulent meningitis. The object of this study is to determine the importance of bacteria and leukocytes as factors causing decrease in CSF sugar both in vivo and in vitro.

MATERIALS AND METHODS

A. Specimens of cerebrospinal fluid (CSF) obtained under aseptic conditions from humans during routine pneumoencephalography were divided into three parts and singly placed at incubator (37° C.), refrigerator (4° C.), and freezer (-20° C.) temperatures. Sugar determinations were done before and 24 hours after placing the specimens at these temperatures. CSF sugar determinations were done by modified Benedict's technique using 1 ml. of CSF.

B. Five milliliter specimens of CSF similarly obtained were inoculated with young cultures of *Micrococcus pyogenes* (var. *aureus*), group A beta hemolytic Streptococcus, *Diplococcus pneumoniae, Neisseria meningitidis,* and *Pseudomonas aeruginosa.* The microorganisms had been grown in trypticase soy broth media. The inoculated CSF specimens were incubated at 37° C. Sugar determinations were done at 0, 8, 18, and 24 hours.

C. Aseptic meningitis in 21 fasting mongrel dogs was established with intracisternal injections of various strengths of streptokinase-dornase solutions† in isotonic saline, 0.75 per cent sterile agar, or aqueous potassium penicillin G, 5,000 units in sterile water. (Fifty milligrams of Nembutal intravenously per 5 pounds of body weight was used for anesthesia.) Inoculation of one of the irritants was done under aseptic conditions, following the removal of 2 to 3 ml. of CSF for sugar determination and cell count. A control blood sugar was obtained as well. Twenty-four hours later, and in 6 dogs, 48 hours later, CSF cell count, CSF sugar, and blood sugar determinations were repeated.

Subsequently specimens of CSF containing the leukocytes were incubated at 37° C. for 24 hours and the sugar content was determined again.

D. Acute purulent bacterial meningitis was produced in 16 fasting mongrel dogs by intracisternal administration of 200 to 2,000 *Pseudomonas aeruginosa* microorganisms‡ (a 10^{-5} or 10^{-6} dilution of an 18-hour culture grown in trypticase soy broth) in 1 ml. of 0.75 per cent sterile agar or broth. Four- to five-milliliter specimens of CSF were removed prior to the inoculation for sugar determination and cell count. A control blood sugar was obtained

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†Some of which was graciously supplied by Lederle Laboratories, Inc., Pearl River, N. Y.

‡A subculture of the Kreich strain of *Ps. aeruginosa* was obtained from Dr. M. Hamburger, University of Cincinnati, College of Medicine, Cincinnati, Ohio. This strain had been used in his studies reported in the J. Infect. Dis. 97: 39, 1955.

as well. Twenty-four, 48, and, in 4 animals, 120 hours later, blood sugar, CSF sugar determinations, and cell counts were repeated. Bacterial counts were done by standard bacterial plate count technique. Because of marked viscosity of CSF, streptokinase (60,000 units)—streptodornase (12,500 units) in 2 ml. of isotonic saline was introduced intracisternally 24 hours after the bacterial inoculation.

Subsequently specimens containing 3 to 4 ml. of sterile dog CSF were inoculated with 200 to 2,000 *Ps. aeruginosa* microorganisms and incubated at 37° C. Bacterial counts and sugar determinations were made 24, 48, and 120 hours after inoculation.

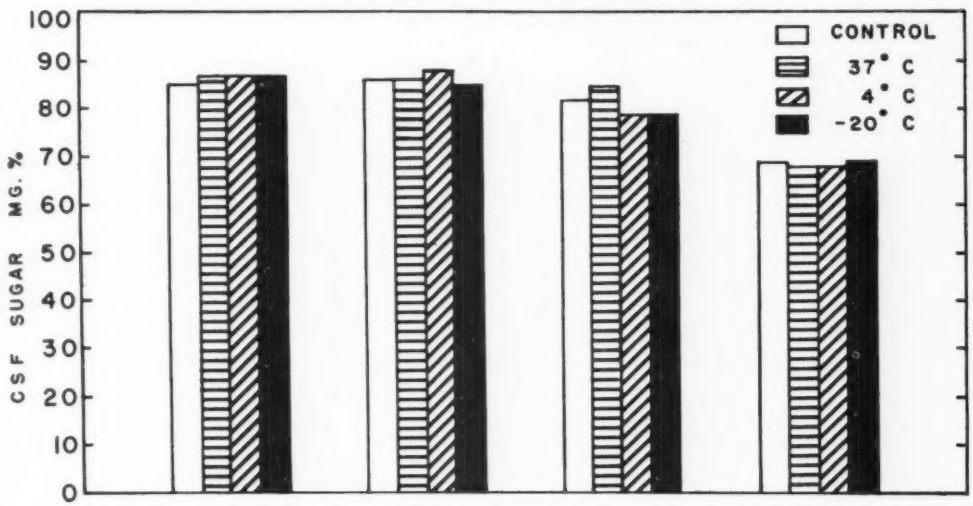

Fig. 1.—Effect of temperature on CSF sugar in vitro in 24 hours.

RESULTS

A. There was essentially no change in the CSF sugar content after singly placing the specimens at 37° C., 4° C., and –20° C. for 24 hours (Fig. 1).

B. Glycolysis was observed in each CSF specimen inoculated with *Micrococcus pyogenes* (var. *aureus*), group A beta hemolytic Streptococcus, and *Diplococcus pneumoniae*. Greatest degree of glycolysis was noted to be at 18 to 24 hours at which time the sugar reduction was noted to be 65 per cent to 0 per cent of the initial level. No glycolysis or bacterial growth was observed in CSF specimens inoculated with *Neisseria meningitidis*. Little or no glycolysis in presence of bacterial growth was observed in CSF specimens inoculated with *Ps. aeruginosa* (Fig. 2).

C. In acute aseptic meningitis caused by various irritants, the resultant pleocytosis at 24 hours ranged from 100 to 13,000 W.B.C./c. mm., 80 per cent of the cells being polymorphs. In most instances, CSF sugars were slightly higher than the blood sugars. There was no significant decrease in the CSF sugar content regardless of the degree of pleocytosis; in fact, in most instances, the concentration of the sugar was essentially at the initial level. Pleocytosis at 48 hours ranged from 12 to 2,000 W.B.C./c. mm. Again there was no significant change in the CSF sugar content observed. (Results of 7 representative animals are shown in Table I.)

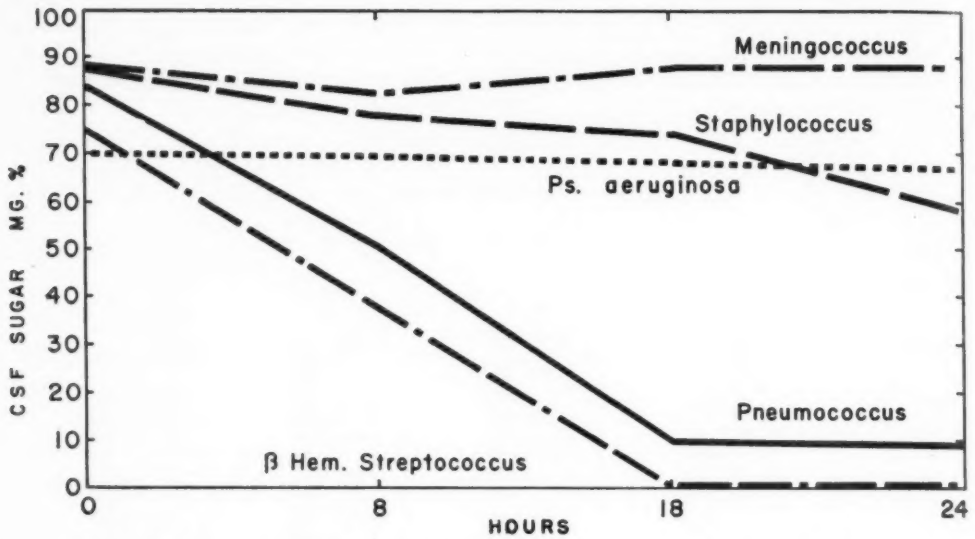

Fig. 2.—Effect of bacterial glycolysis on CSF sugar in vitro at 0, 18, and 24 hours.

TABLE I. EFFECT OF INTRACISTERNAL ADMINISTRATION IN DOGS OF STREPTOKINASE-DORNASE SOLUTION IN ISOTONIC SALINE ON THE SUGAR CONTENT AND PRODUCTION OF PLEOCYTOSIS IN 24 AND 48 HOURS

	CONTROL SUGAR MG. %		AT 24 HOURS			AT 48 HOURS		
			SUGAR MG. %		W.B.C./	SUGAR MG. %		W.B.C./
DOG NO.	BLOOD	CSF	BLOOD	CSF	C. MM.	BLOOD	CSF	C. MM.
1	74	85	74	82	100	68	74	12
2		87		84	1,620			
3	70	76	81	86	2,500	70	76	60
4	72	85	80	89	3,200			
5	73	82	81	83	6,900	76	85	2,000
6	84	93	82	83	7,800	92	81	810
7	80	98	78	90	13,000			
Av.	72.2	86.6	79	86.6		76.5	79	

TABLE II. EFFECT OF LEUKOCYTES ON THE DECREASE IN THE SUGAR CONTENT IN CSF IN VITRO

	CSF		
	SUGAR MG. %		W.B.C./C. MM.
DOG NO.	CONTROL	AT 24 HOURS	
8	72	77	8
1	85	82	100
9	99	93	212
10	75	69	450
11	92	75	810
12	85	48	2,000
13	86	52	2,500
14	96	31	6,000
6	93	29	7,800
7	98	13	13,000

When sterile specimens of CSF containing the leukocytes were incubated at 37° C. for 24 hours, a drop in the sugar content occurred in each instance in which the number of leukocytes present was greater than 450/c. mm. Generally, the greater the pleocytosis, the greater was the drop in the sugar content. (Results of 10 representative CSF specimens containing leukocytes are seen in Table II.)

D. In acute purulent meningitis produced by *Ps. aeruginosa*, the resultant pleocytosis in the first 24 hours varied from 2,070 to 15,000 W.B.C./c. mm., 90 to 95 per cent of the leukocytes being polymorphs; many were clumped. The bacterial colony counts ranged from 0 to 610,000/ml. of CSF. In 7 of the 16 dogs, bacterial counts above 65,000/ml. were obtained. In 2 animals, the CSF sugar content dropped 80 per cent of the initial level, and in all instances, it dropped more than 30 per cent if viable microorganisms were cultured. (Results of 8 representative animals are seen in Table III.) It was evident that the larger the number of viable microorganisms, the greater was the pleocytosis and the lower was the CSF sugar content. Forty-eight hours after the bacterial inoculation and twenty-four hours after streptokinase-dornase solution was introduced intrathecally, the pleocytosis increased to 2,900 to 36,000 W.B.C./c. mm., bacterial counts ranged from 0 to 18,000/ml. of CSF, and the sugar content in most cases showed a slight increase over that seen at 24 hours after the bacterial inoculation. (Results of 8 representative animals are seen in Table IV.)

TABLE III. RESULTS OF CSF SUGAR CONTENT AND BACTERIAL COUNTS 24 HOURS FOLLOWING INTRACISTERNAL *Ps. aeruginosa* INOCULATION SUSPENDED IN 0.75 PER CENT AGAR OR BROTH

DOG NO.	IRRITANT	NO. BACTERIA INOCULATED	CONTROL SUGAR MG. % BLOOD	CONTROL SUGAR MG. % CSF	24 HOURS NO. BACTERIA CULTURED/ML.	24 HOURS SUGAR MG. % BLOOD	24 HOURS SUGAR MG. % CSF	W.B.C./C. MM.
11	Agar	255	73	70	65,280	72	46	8,000
12	Agar	255	73	73	87,680	88		8,510
13	Agar	2,740	87	86	224,800	90	60	7,200
14	Agar	2,740	63	72	136,000	85	42	6,480
15	Agar	320	87	76	610,000	79	18	13,000
16	Agar	320	69	79	606,000	95	25	15,000
17	Broth	300	65	80	0	77	92	2,070
18	Broth	300	79	83	0	73	88	4,536

TABLE IV. RESULTS OF CSF SUGAR CONTENT AND BACTERIAL COUNTS 48 HOURS FOLLOWING INTRACISTERNAL *Ps. aeruginosa* INOCULATION SUSPENDED IN 0.75 PER CENT AGAR OR BROTH AND 24 HOURS AFTER STREPTOKINASE-DORNASE (60,000 UNITS AND 12,500 UNITS) ADMINISTRATION

DOG NO.	IRRITANT	NO. BACTERIA INOCULATED	CONTROL SUGAR MG. % BLOOD	CONTROL SUGAR MG. % CSF	48 HOURS NO. BACTERIA CULTURED/ML.	48 HOURS SUGAR MG. % BLOOD	48 HOURS SUGAR MG. % CSF	W.B.C./C. MM.
11	Agar	255	73	70	130	77	51	2,900
12	Agar	255	73	73	6,460	79	47	17,280
13	Agar	2,740	87	86				
14	Agar	2,740	63	72	600	86	37	10,800
15	Agar	320	87	76	1,000	84	33	22.000
16	Agar	320	69	79	18,000	91	38	36,000
17	Broth	300	65	80	0	80	84	4,700
18	Broth	300	79	83	0	79	97	990

At 120 hours after the bacterial inoculation the CSF remained viscid, yellow, and cloudy. A sufficient amount of CSF for study was obtained in one dog only. It contained 5,100 W.B.C./c. mm., a bacterial count of less than 100,000/ml., and a sugar content 50 per cent of the initial level. At autopsy (2 dogs at 5 days and 2 dogs at 7 days after the bacterial inoculation) the animals showed a viscid yellow exudate around the areas of the fourth ventricle, base

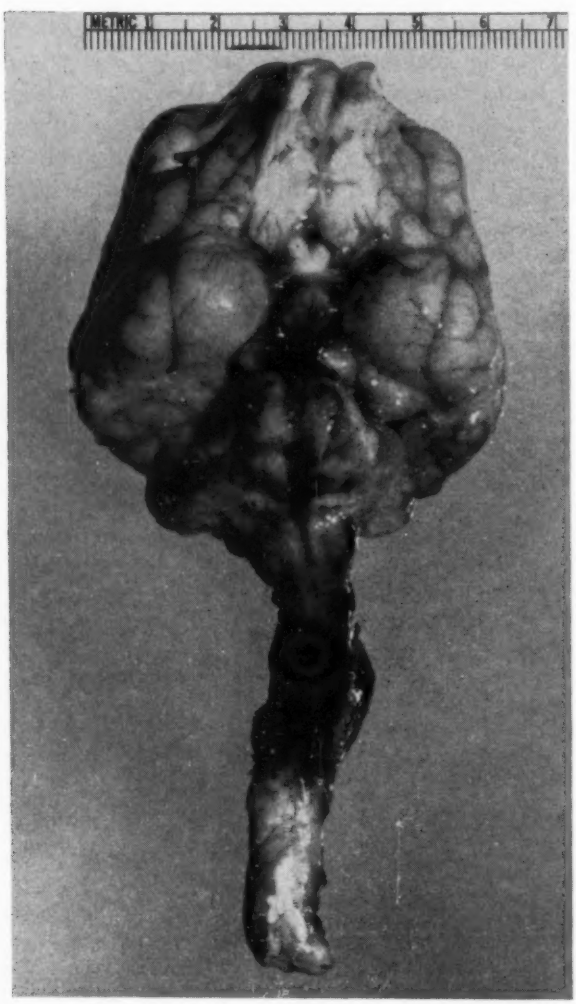

Fig. 3.—See text.

of the brain, and upper spinal cord; the blood vessels were prominent and engorged; there were hemorrhagic areas seen around the upper spinal cord (Fig. 3). In one of the 16 dogs the infection was overwhelming, causing coma and death on the fifth day. One dog developed hemiplegia within 48 hours. Disease in the remaining 14 animals was self-limited, handled by the animal itself without antibacterial therapy.

It should be noted that when 200 *Ps. aeruginosa* microorganisms were introduced into 1 ml. of broth and inoculated intracisternally, pleocytosis of at least 4,500 W.B.C./c. mm. was produced, but no sugar decrease was seen or positive CSF culture obtained (Tables III and IV).

When 3 to 4 ml. specimens of sterile dog CSF were inoculated with 200 to 2,000 *Ps. aeruginosa* microorganisms in vitro, the bacterial counts reached 58,000,000,000/ml. 24 hours later, but no decrease in the sugar content was seen. Both the number of viable bacteria and sugar concentration decreased during the next few days. (Results of 6 representative CSF specimens are seen in Table V.)

TABLE V. RESULTS OF CSF SUGAR CONTENT AND BACTERIAL COUNTS FOLLOWING IN VITRO INOCULATION OF CSF WITH *Ps. aeruginosa*

	CONTROL		24 HOURS		48 HOURS		72 HOURS		120 HOURS	
DOG NO.	SUGAR MG. %	NO. BACTERIA /ML.	SUGAR MG. %	NO. BACTERIA/ ML. (× 1,000,000)	SUGAR MG. %	NO. BACTERIA/ ML. (× 1,000,000)	SUGAR MG. %	NO. BACTERIA/ ML. (× 1,000,000)	SUGAR MG. %	NO. BACTERIA /ML.
11	70	255	68	1,113						
12	73	255	69	1,280						
13	86	2,740	85	58 800	76	804	46	< 10		
14	72	2,740	81	57,000	65	1,650	24	< 10		
15	76	320	79	10,000	14	400			11	<100,000
16	79	320	79	10,000	56	300			16	<100,000

It was evident that a decrease in the sugar content was observed only if viable microorganisms were subsequently cultured in vitro, and that much higher counts of microorganisms were necessary to observe the same degree of glycolysis in vitro as found in vivo.

DISCUSSION

From these data it is evident that, in vitro, both microorganisms and leukocytes are capable independently of causing glycolysis. Reducing substances in the CSF were first noted and studied by Deschamps and Bussy in 1852.[1] This was proved to be glucose by Denigés in 1896.[2] Recently, Eastham and Keay[3] have demonstrated by paper chromatography at least three reducing substances in the CSF: glucose, fructose, and glucosamine.

It is known that most microorganisms utilize carbohydrates for energy and growth as is confirmed by our observations in vitro. Presumably they could be the cause of hypoglycorrhachia in acute meningeal infections. However, in 1950, Goldring[4] suggested that the number of microorganisms needed to produce a decrease in the CSF sugar in vitro is much greater than that usually seen in acute purulent meningitis. Our observations with *Ps. aeruginosa* in vivo and in vitro confirm this. In addition, it becomes difficult indeed to explain the low sugar values reported many times in tuberculous meningitis when the organism is frequently difficult to observe on smear and to culture, thus suggesting the presence of very few microorganisms. Consequently, the mere presence of bacteria in the CSF is not enough to account for the decrease of the CSF sugar in vivo.

The next question is the ability of leukocytes to cause glycolysis. In 1911, Grafe[5] demonstrated that leukocytes consume oxygen. One year later Levene and Meyer[6] found that leukocytes split sugar and produce lactic acid. MacLean and Weir[7] showed that leukocytes utilize sugar 200 to 1,000 times more rapidly than red blood cells, that polymorphs utilize sugar 5 times more rapidly than mononuclears. Guest and Mackler[8] have stated that at 37° C. in vitro blood can utilize 15 mg. glucose/100 ml./1 hr. We have demonstrated as well that incubation of sterile CSF with leukocytes causes the sugar to decrease. However, regardless of the degree of pleocytosis we were unable to detect glycolysis in aseptic meningitis in dogs. This would suggest that the body is capable of replenishing the amount of glucose utilized by the leukocytes in vivo, the glucose level remaining in physiologic range at all times. Thus, it is observed that, in vivo, leukocytes are not the cause of hypoglycorrhachia.

If the presence of bacteria or leukocytes alone in vivo is inadequate to cause a decrease in the CSF sugar, there should be other contributing factors. The problem of blood-brain barrier in passage of sugar and other substances and increased utilization of glucose by cerebral cells may play a part. The blood-brain barrier (BBB) has been studied with vital dye technique in the past and with radioactive isotopes more recently.[9-13] This barrier exists in the lowest vertebrates. It has been observed that the tracer uptake by brain decreases after birth and at 7 weeks of age it is no different from the adult. The blood-brain barrier seems to exist even a few hours after death. The anatomical location of the barrier by modern techniques seems to be localized in the inner wall of the cerebral capillary membrane and the inner wall of the choroid plexus. This barrier is very selective for different substances. Greenberg[10] has found that ionic permeability to potassium is greatest, reaching 50 per cent of plasma level in 30 minutes, then sodium reaching 60 per cent in one hour, and phosphorus reaching 23 per cent in 2 hours. CSF glucose usually is found to be about 60 per cent of the blood glucose level in the fasting normal human. Doubling the blood glucose level will eventually cause doubling of the CSF glucose as well, but the delay in rise may be 2 to 3 hours and return to normal levels 6 to 7 hours.[14] Unfortunately little if any work has been done regarding the BBB studies in acute meningeal infection as far as glucose and ionic permeability are concerned.

CONCLUSIONS

Our data provide the following information.

1. Incubation, refrigeration, or freezing of sterile cell free CSF specimens does not change the sugar content.

2. Microorganisms certainly are capable of utilizing sugar in CSF in vitro.

3. Regardless of the degree of pleocytosis, the sugar values in aseptic meningitis do not change. Reduction of CSF sugar is seen when CSF with large numbers of leukocytes is incubated in vitro.

4. In acute meningitis produced by *Ps. aeruginosa* the reduction of the sugar content cannot be explained on bacterial and cell glycolysis alone. It is

suggested that a change in the BBB and perhaps an increase in the utilization of glucose by the cerebral cells may be responsible to a large degree for the hypoglycorrhachia seen in acute bacterial meningitis.

We are indebted to Mrs. Arckie Canton and her assistants for the technical help in blood and CSF sugar determinations. We are deeply grateful to Dr. Paul Bunn for his most valuable suggestions and continued interest in this study.

The illustrations and tables in this paper are from the Medical Illustration Laboratory, Syracuse V. A. Hospital, Syracuse, N. Y.

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HUMORAL REGULATION OF ERYTHROPOIESIS
III. EFFECT OF EXPOSURE TO SIMULATED ALTITUDE*

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IT HAS been demonstrated in a number of laboratories[1-8] that plasma of animals with posthemorrhagic anemia contains a substance capable of stimulating erythropoiesis which has been referred to as hemopoietine or erythropoietine. There is also indirect evidence to suggest that a similar mechanism is responsible for the observed polycythemia in animals and man with a lowered arterial oxygen tension.[9, 10, 14] Direct evidence for the presence of erythropoietine in the plasma under these circumstances has not been presented. It is the purpose of this communication to present data indicating that animals exposed to simulated altitudes elaborate erythropoietine but that the effectiveness of this material in stimulating erythropoiesis is limited by the degree of bone marrow oxygenation.

METHODS AND MATERIAL

Sublethally irradiated rats were used for the assay.[6, 11] Female Sprague-Dawley rats weighing from 135 to 160 grams were randomly assigned to 4 or 5 groups. These animals received 160 to 190 roentgens from a 2.5 MEV Van de Graaff generator at an average dose rate of 50 roentgens per minute, measured with a Bendix ionization chamber known to be air equivalent at this energy.† Immediately after irradiation, test plasma was given or the animals were bled or exposed to altitude as the experiment dictated. Forty-eight hours after irradiation, plasma-bound Fe^{59} was given intravenously and 48 hours thereafter a sample of blood was obtained by cardiac puncture to determine the per cent of the injected dose of Fe^{59} which had been incorporated into the red cells. In determining the per cent of iron incorporation, a blood volume figure of 7 per cent of the preirradiation body weight has been used in the past.[6, 11] It subsequently was determined by the use of Fe^{59}-labeled red cells that in this colony of rats the blood volume of animals that were irradiated and remained at ground level was equivalent to 6.1 ± .54 per cent (s.d.) of the preirradiation body weight or 6 ± 0.6 per cent (s.d.) of the 96-hour postirradiation body weight. In those animals which had been exposed to altitude continuously for 96 hours following irradiation, the blood volume was 7.2 ± 1.48 per cent (s.d.) of the postexposure weight. Presumably these differences are the result of a marked loss of body weight by the altitude exposed animals. The average weight loss following a 96-hour exposure at 22,000 feet is 40 to 50 grams.

Animals were exposed to simulated altitudes in a decompression chamber.‡ Throughout this paper, altitude is referred to in terms of feet above sea level. The barometric pressure at these levels may be obtained from a number of standard sources. For example, at 22,000 feet the barometric pressure is 320 mm. Hg.

From The National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

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*References 6 and 11 were the first two papers in this series.

†Particular thanks are due to Dr. Howard Andrews of the National Cancer Institute for the use of his irradiation facilities, and the measurements of field uniformity and delivered dose which were critical for the reproducibility of these experiments.

‡We are indebted to Drs. Paul Altland and Heinz Specht of the National Institute of Arthritis and Metabolic Diseases, who generously provided these facilities.

"Anemic" plasma was prepared by bleeding donor rats 2.1 per cent of the body weight. Twenty-four hours thereafter the animals were bled again and the separated plasma served as anemic plasma. "Altitude" plasma was obtained from normal rats which had been exposed at the desired level of altitude in a decompression chamber for 24 hours.

RESULTS

Plasma from animals exposed at a simulated altitude of 22,000 or 24,500 feet produced a significant increase in erythropoiesis in the sublethally irradiated animal (Fig. 1, Table I). In each of four experiments performed, the recipients of plasma from donors exposed at these levels of altitude incorporated 20-30 per cent more radioactive iron than did irradiated but otherwise untreated controls. The degree of stimulation produced by plasma from donors exposed at these levels was similar to that achieved with anemic plasma and significantly greater than that obtained with normal plasma (Fig. 1).

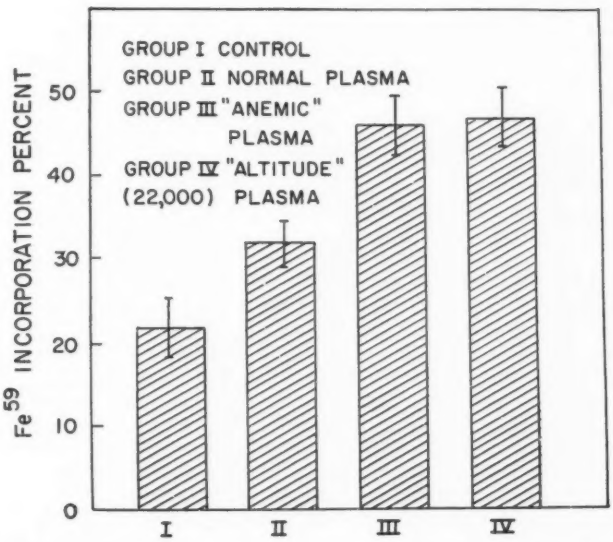

Fig. 1.—The effectiveness of "altitude" plasma in stimulating erythropoiesis is compared with that of "anemic" and normal plasma in the sublethally irradiated animal. Controls are irradiated but otherwise untreated animals. Fe^{59} values represent the per cent of the injected dose incorporated into the peripheral red cell 48 hours after injection ± a single standard error.

While in the particular experiment shown in Fig. 1 the recipients of normal plasma incorporated a significantly greater amount of radioiron than did the controls, this has not been a consistent finding under the conditions of these experiments. A significant increase in iron incorporation was observed in only one of four experiments where erythropoiesis in the irradiated recipients of normal plasma was directly compared with that in irradiated controls.[6]

The effectiveness of altitude plasma in stimulating erythropoiesis depended upon the level of altitude at which the donor rat had been exposed (Table I). There was some increase in iron incorporation produced by plasma from donors exposed at all altitude levels studied (14,000-24,500 feet), but plasma from donors exposed at higher altitudes produced a substantially greater response. Thus, recipients of plasma from donors exposed at 14,000

feet had an increase of 9 per cent in the average Fe^{59} incorporation in contrast to a 25 per cent increase observed in animals receiving plasma from donors exposed at 22,000 feet.

TABLE I. EFFECT OF PLASMA FROM DONORS EXPOSED TO SIMULATED ALTITUDE ON Fe^{59} INCORPORATION IN THE SUBLETHALLY IRRADIATED RAT

ALTITUDE (FEET)	CONTROL	ALTITUDE PLASMA	P*
14,000	24 ± 2.6 (7)†	33 ± 2.3 (5)	$< .05$
16,500	27 ± 3.1 (7)	35 ± 2.9 (7)	$< .1 > .05$
19,500	27 ± 2.3 (6)	35 ± 0.5 (6)	$< .01$
22,000	22 ± 3.4 (5)	46 ± 3.4 (5)	$< .01$
24,500	16 ± 2.6 (7)	44 ± 1.8 (5)	$< .01$

*P refers to probability that observed differences between control groups and those receiving "altitude" plasma occurred by chance.

†Fe^{59} values given represent per cent of total injected dose present in circulating red cells 48 hours after injection ± a single standard error computed as $\frac{\text{Range}}{K_n \sqrt{n}}$.[13] Number of animals in each group is included in parentheses.

The pattern of response of animals exposed to simulated altitude for 96 hours following sublethal irradiation differed significantly from that of the irradiated animal which received altitude plasma but remained at ground level. The results of iron incorporation in rats exposed to altitude following irradiation are shown in Table II. The levels of altitude varied from 16,500 to 24,500 feet. A modest stimulation was achieved in those exposed at 16,500 feet as evidenced by an increase in iron incorporation to 37 per cent from the control value of 27 per cent. The greatest effect was noted in those animals exposed at 19,500 feet. At this level animals incorporated 45 per cent of the injected dose of radioiron in contrast to the 24 per cent observed in the irradiated controls remaining at ground level. Exposure at higher altitudes, however, reduced the magnitude of the erythropoietic response. Thus, animals exposed at 22,000 feet incorporated only 29 per cent of the injected dose of iron.

TABLE II. EFFECT OF EXPOSURE TO SIMULATED ALTITUDE ON Fe^{59} INCORPORATION IN THE SUBLETHALLY IRRADIATED RAT

LEVEL OF ALTITUDE*	CONTROL	ALTITUDE	P*
16,500	27 ± 3.1 (7)†	36.6 ± 4.1 (7)	$= .05$
19,500	24 ± 2.6 (7)	45 ± 3.1 (7)	$< .01$
22,000	21 ± 1.96 (7)	29 ± 2.3 (7)	$< .05$
24,500	16 ± 2.6 (7)	32 ± 2.4 (12)	$< .01$

*P refers to probability that observed differences between control groups and those exposed to altitude occurred by chance.

†Fe^{59} values given represent per cent of total injected dose present in circulating red cells 48 hours after injection ± a single standard error computed as $\frac{\text{Range}}{K_n \sqrt{n}}$.[13] Number of animals in each group is included in parentheses.

Since it has been demonstrated that postirradiation bleeding produces a striking stimulation of erythropoiesis[11] it was considered of interest to study the combined effect of altitude and bleeding. Rates of erythropoiesis, therefore, were studied in animals which were bled immediately following irradiation and thereafter exposed continuously at altitude for 96 hours, the duration of the experiment. These effects were determined in animals exposed

at levels varying from 16,500 to 22,000 feet. In Table III these values are presented together with values for animals which were bled and remained at ground level. In a typical experiment, bleeding immediately following irradiation increased the iron incorporation from a control value of 21 per cent to 45 per cent. However, in a group of animals bled immediately following irradiation and then exposed continuously at 22,000 feet, only 24 per cent of the injected dose of radioactive iron was incorporated. There was a similar reduction in the response to hemorrhage when sublethally irradiated animals were placed at 19,500 feet immediately after bleeding. At 15,000 feet there was some decrease in the response to bleeding observed in animals exposed but the differences were not statistically significant (Table III). It was apparent from these studies that the erythropoietic response evoked by bleeding following irradiation could be suppressed by the induction of severe hypoxia.

TABLE III. EFFECT OF BLEEDING AND EXPOSURE TO SIMULATED ALTITUDES ON Fe^{59} INCORPORATION IN THE SUBLETHALLY IRRADIATED RAT

EXPOSURE LEVEL IN FEET	CONTROL	BLED	BLED AND EXPOSED TO ALTITUDE	P*
16,500	25 ± 1.6 (5)†	45 ± 6.7 (7)	36 ± 3.9 (7)	n. s.
19,500	11.3 ± 2.6 (7)	44 ± 4.2 (6)	30 ± 2.3 (9)	< .01
22,000	21 ± 2 (7)	43 ± 3.8 (6)	25 ± 1.8 (6)	< .01

*P refers to probability that the observed differences between those groups which were bled and remained at ground level and those groups which were bled and exposed to simulated altitudes occurred by chance.

†Fe^{59} values given represent per cent of total injected dose present in circulating red cells 48 hours after injection ± a single standard error computed as $\frac{\text{Range}}{K_n \sqrt{n}}$.[13] Number of animals in each group is included in parentheses.

DISCUSSION

The data demonstrate that exposure to altitude, like hemorrhage, evokes the release of a humoral factor capable of stimulating erythropoiesis. The effectiveness of plasma collected from donors exposed at higher altitudes, 22,000 or 24,500 feet, was substantially greater than that from donors exposed at altitudes of 14,000-19,500 feet. Thus, the amount of erythropoietine liberated depended upon the exposure level of the animals. The data are consonant with previous reports which indirectly indicated that the polycythemia incident to hypoxia was the result of a humoral influence on the marrow.[9, 10] Reismann[9] demonstrated increased red cell production in both parabionts when one breathed room air and the second breathed air with a lowered oxygen content. In a patient with polycythemia, secondary to regional hypoxia, increased erythropoietic activity was observed in those areas of the bone marrow where the arterial oxygen saturation was normal.[10]

The use of the sublethally irradiated animal as a test animal in these experiments is of considerable importance. Whereas in normal animals multiple injections of anemic plasma are required to evoke a demonstrable increase in red cell production, only a single injection is necessary in the sublethally irradiated animal. The increased sensitivity of these animals to erythropoietine may be the consequence of a diminution in a normally occurring inhibitor.

Furthermore, moderate increases in red cell production are more readily quantified by measurement of Fe^{59} incorporation than by other currently available methods.

Of particular interest is the erythropoietic response of the sublethally irradiated rat when placed at simulated altitudes. While there was some stimulation of erythropoiesis at all levels (16,500-24,500 feet) the maximum response was achieved in animals placed at a simulated altitude of 19,500 feet. Moreover, the previously described stimulation of erythropoiesis afforded by bleeding animals immediately after irradiation[11] was modified by the severe marrow hypoxia incident to exposure to altitude.

It is to be noted that the plasma from donors exposed at higher altitudes when given to test animals at ground level produced a more effective stimulation of erythropoiesis than did exposure of the test animal at the same level of altitude. At 24,500 feet, control animals incorporated 16 per cent of the injected dose of radioiron while those exposed at altitude incorporated 31 per cent of the administered iron. In striking contrast, in the same experiment recipients of plasma from donor rats exposed at 24,500 feet incorporated 46 per cent of the injected dose. These studies demonstrate that the diminished erythropoietic response at higher altitudes is the consequence of the inability of the marrow to respond to the erythropoietic stimulus rather than a decreased production of erythropoietine. Presumably these observations are explained by the fact that red cell precursors have a primarily oxidative metabolism and hypoxia, when severe, limits the response to the erythropoietic stimulus. In keeping with these observations are the previous reports of Talbot and his associates[12] during an Andean expedition. These investigators reported fragmentary evidence that on ascent to 20,000 feet, the erythropoietic response to hypoxia was diminished. In one patient with polycythemia secondary to regional hypoxia, the erythropoietic activity was diminished in the iliac crest where the arterial pO_2 of 35.8 mm. Hg was lower than that which is observed at altitudes of 20,000 feet.[10] It was suggested that the apparent suppression of erythropoiesis in this case was the consequence of local anoxia, which was severe enough so that the red cell precursors could not respond to the primary humoral stimulus. The results reported herein would support such a hypothesis.

SUMMARY

Data demonstrate that exposure to altitude results in the release into the plasma of an erythropoietic principle similar to that previously demonstrated in posthemorrhagic anemia. It further has been demonstrated that the response of animals at altitude to the erythropoietic factor may be limited when hypoxia of the bone marrow becomes severe. The latter presumably is related to the primarily oxidative metabolism of the red cell precursors which require adequate oxygenation for a maximal response to the erythropoietic stimulus.

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ADDENDUM

Since submission of this manuscript, Gray and Erslev (Proc. Soc. Exper. Biol. & Med. **94**: 283, 1957) have reported that plasma from rabbits exposed for 48 hours to 10 per cent O_2 produced a significant increase in reticulocytes when given daily for 4 days to normal recipients. The magnitude of response of the recipients of hypoxic plasma was equivalent to that of those animals exposed to 10 per cent O_2.

PAPER ELECTROPHORETIC SURVEY OF HEMOGLOBINS OF AMERICAN INDIANS

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FAMILY studies undertaken during the course of a previously published investigation of sickle-cell-hemoglobin C disease[1] indicated the presence of American Indian ancestry in one of the kinships. In this family the mother had sickle-cell trait and the father's blood contained AC hemoglobin. Although the father and his children exhibited Negroid features, he stated that his mother was half Cherokee Indian and half white. His father was described as a "Blanket Indian," his paternal grandfather a "Black Hawk Indian," but he knew nothing about his paternal grandmother. The family previously lived near Richmond, Virginia. Another family from Columbia, Missouri, in which both parents had hemoglobin C trait, likewise gave a history of Indian ancestry which they believed to be Cherokee. Still another patient with sickle-cell-hemoglobin C disease gave a vague history of Indian ancestry in his father's family which harbored the hemoglobin C gene.

Therefore, it seemed possible at the time this investigation was first begun (April 26, 1954) that hemoglobin C might be indigenous to the American Indian and that it might have been introduced into the American Negro race by miscegenation of the races. Consequently, a survey of the hemoglobin types of a group of American Indians in Oklahoma was conducted. In none of the 259 individuals studied was hemoglobin C detected. Three were found to have the sickle-cell trait. In none of the 3, however, was it possible to completely exclude the possibility of Negro ancestry.

METHODS

Blood samples were drawn from 259 Oklahoma Indians. At the Kiowa Indian Hospital at Lawton, Oklahoma, 150 samples were procured predominately from western plains Indians. An additional 109 samples were collected from patients of the Hastings Indian Hospital and students at the Sequoya Indian School of Tahlequah, Oklahoma. Most of the individuals from the latter institutions were descendants of the so-called "Five Civilized Tribes," the Cherokees, Choctaws, Chickasaws, Creeks, and Seminoles who originated in the southeastern states.

Three milliliter samples of oxalated venous blood were collected and transported to St. Louis in an ice chest. Paper electrophoresis was performed with the use of glass plates and barbital buffer at pH 8.8 and 0.06 ionic strength as previously described.[2] On each specimen the hemoglobin concentration was determined and sodium metabisulfite sickle-cell preparations and dried blood smears for Wright staining were made.

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RESULTS

The tribal distribution of the Indians examined and the degree of Indian ancestry claimed by them are shown in Table I. These tribes can be grouped into 7 linguistic families. Eighty-seven of the Indians studied were of Cherokee descent. Of these, one person who stated that he was half Cherokee and half white was found to have a positive sickle-cell preparation and an A-S hemoglobin electrophoresis pattern. One $^{13}/_{16}$ degree Creek and an allegedly "full-blooded" ($^{4}/_{4}$ degree) Wichita also had sickle-cell trait. The remaining

TABLE I. TRIBAL DISTRIBUTION OF INDIANS EXAMINED AND DEGREE OF INDIAN ANCESTRY

	DEGREE OF INDIAN ANCESTRY					
TRIBE	?	1/4	1/2	3/4	4/4	TOTAL
Iroquois Family						
Cherokee	8	2	18	13	46	87
Muskhogean Family						
Chickasaw	1		1	1	1	4
Choctaw	4	3	3	5	18	33
Creek	4		3	5	19	31
Seminole	1		2	2	3	8
					Total	76
Caddo Family						
Wichita			1		5	6
Pawnee					3	3
Caddo		1			2	3
					Total	12
Siouan Family						
Sioux			1	1	1	3
Omaha	1					1
Osage				2		2
Ponca					1	1
Quapaw	1		1			2
					Total	9
Déné Family						
Navaho				1	6	7
Apache					2	2
					Total	9
Uto-Aztecan Family						
Aztec			1			1
Jemez			1			1
Comanche	7	1	4	1	14	27
Hopi					1	1
Kiowa	2	1	3	1	6	13
Ute				1		1
Taos					1	1
					Total	45
Algonquin Family						
Arapahoe	1				1	2
Cheyenne	2				1	3
Delaware			1			1
Gros Ventre				1		1
Kickapoo					1	1
Sauk and Fox	1					1
Shawnee					1	1
					Total	10
Unknown						11
Total						259

individuals, distributed among 31 tribes, exhibited no hemoglobin abnormalities. In 3 persons the hemoglobin types were normal and the blood films showed hypochromic microcytic erythrocytes. The remainder of the hematological examinations conducted were normal.

DISCUSSION

The family histories obtained in the Negro patients with C hemoglobin suggested that hemoglobin C might be indigenous to the American Indian or, at least, to the Cherokee. Anthropologists have collected considerable data to indicate that the American Indian may have migrated in antiquity to this continent from Asia over a postulated Manchurian-Alaskan land bridge. In view of the large number of abnormal hemoglobins found in southern Asia,[3-6] it seemed reasonable to study the hemoglobins of the American Indian for other components in addition to hemoglobin C. Since hemoglobin C has been found in about 2 per cent of the North American Negro population in large surveys,[7, 8] one would anticipate its incidence to be considerably higher in the Indian if it had been introduced to the Negro race by miscegenation with the Indian. Since this survey was undertaken, the discovery of hemoglobin C in about 15 per cent of some West African tribes[9] makes the absence of hemoglobin C in the American Indian somewhat less surprising.

The racial homogeneity of the American Indian is still conjectural, and for this reason the tribes sampled in this survey were grouped according to a linguistic classification (Table I), which also correlates well with their aboriginal geographic distribution.[10, 11] Although only small numbers of individuals were examined from any single tribe, the grouping of the tribes into linguistic families lends more validity to the findings. The Cherokees, who were the largest tribe examined, originated in western North Carolina, Virginia, South Carolina, Georgia, and eastern Tennessee.[12] Most of them were moved to Oklahoma by the Federal Government in the historic "Trail of Tears" in 1838 and thus many now live in Oklahoma.[13] In addition, 76 persons were descendants of the Muskhogean Family: the Chocktaws, Chickasaws, Creeks, and Seminoles. These originated in the southeastern states of Alabama, Mississippi, Louisiana, Georgia, and Florida and were likewise moved to Oklahoma by the Federal Government in the early nineteenth century.[13] Thus the 87 Cherokees and the 76 descendants of the Muskhogean family comprise a rather large racially similar sample of the aboriginal Indian of the southeastern United States. Among these Indians only 2 cases of sickle-cell trait were found.

The remaining individuals of this series are grouped in the Caddo Family of northeastern Texas and Arkansas, the Déné from Arizona and New Mexico, the Siouan and Uto-Aztecan also from Texas and New Mexico, and a few of the Algonquin from the north central states. Eighty-five persons were examined from this group and only one person, an alleged full-blooded Wichita, had the sickle-cell trait, a finding which cannot be considered significant.

The results of this survey suggest that the Indians of the western plains states and of the southeastern United States do not possess a genetically determined abnormal hemoglobin. The one per cent incidence of sickle-cell trait is probably attributable to miscegenation with the American Negro. Finally, it is suggested that it may be of some interest to conduct similar surveys of other Indian tribes, particularly, those inhabiting the western coast in order to evaluate them for the presence of an abnormal hemoglobin.

SUMMARY

Blood samples were collected from approximately 150 American Indians from the plains states and from another 100 Indians originating in the southeastern United States. Of the samples examined by electrophoresis, only 3 cases of sickle-cell trait were found. It is thought that these cases of sickle-cell trait are probably attributable to miscegenation with the American Negro.

The author is grateful to A. I. Chernoff, M.D., under whose guidance this survey was conducted, as well as to S. NaNakorn, M.D., and V. Minnich, M.S., for technical assistance; to Dr. Syble Adams of the United States Government, Kiowa Indian Hospital, Lawton, Okla., Dr. J. M. Gowdy of the Hastings Indian Hospital, and Mr. Jack McCarthy of the Sequoya Indian School, Tahlequah, Okla., for making available their facilities for the collection of this series of blood samples.

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CHOLESTEROL IN SOME HEMATOLOGIC DISORDERS: A COMPARISON OF FINDINGS IN SERUM AND TISSUES

With Special Reference to Lymphoma

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CHANGES of content and distribution of serum proteins and cholesterol have been reported in lymphoma.[1] There is, in this disease, constant and considerable reduction of total cholesterol and esters in serum. Also, protein-bound cholesterol (and lipoproteins of serum) are increased in the albumin and α_1-globulin fraction and decreased in the β-globulin fraction. The question then arose whether these results, which were significant enough to be diagnostic in many instances, could be correlated to similar changes occurring in tissues.

Severe untreatable hemolytic anemia, hypersplenic thrombocytopenia, or other considerations have advised splenectomy in some of our patients with hematologic disorders. Splenic tissue and, in rare instances, isolated lymphoma tissue then became available for chemical studies under ideal conditions of preservation of proteins and lipids. An additional, less satisfactory, source of lymphoma spleen and tissue was autopsy material obtained shortly after death. Modifications in content and partition of cholesterol and certain proteins in splenic tissue and their relation to changes in serum were studied.

MATERIALS

Twenty-eight spleens were obtained either at surgery or shortly (2 to 4 hours) after death (Table I). Hodgkin's tissue was taken from organs where, occasionally, well-defined easily separable areas were found (Fig. 1). In many instances, serum was collected from the patients at the same time. Patients with lymphoma and leukemia were newly discovered cases or cases in a state of clinical relapse. Changes to be described were found in treated and untreated cases alike, provided the patient had evidence of clinical relapse and activity of the disease. This finding removed a major objection in the interpretation of our studies.

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METHODS

1. *Preparation of Splenic Tissue for Lipidic Extraction.*—Immediately after collection, spleens were perfused repeatedly through the splenic artery stump with cold saline to remove most of the blood. When washing fluid was no longer tinged with blood, spleens were frozen at −20° C. In a few instances, as indicated in the tables with asterisks, a different, perhaps more valid technique was used. Surgical spleens were washed immediately with cold saline solution and immersed in liquid nitrogen for instantaneous freezing. The organ was then kept at −20° C. Spleens were slowly thawed at the moment of use. Aliquots were transferred to test tubes and gently squeezed with glass rods to obtain a pulp. Four parts in volume of cold saline then were added to the pulp. After mixing, tubes were centrifuged at 3,200 r.p.m., 30′/4° C. Supernatant, containing large amounts of hemoglobin, was discarded; the residue was resuspended in saline. The process was repeated 5 times to remove as much hemoglobin and plasma as possible. In all cases, no hemoglobin could be detected by chemical methods after the last washing.

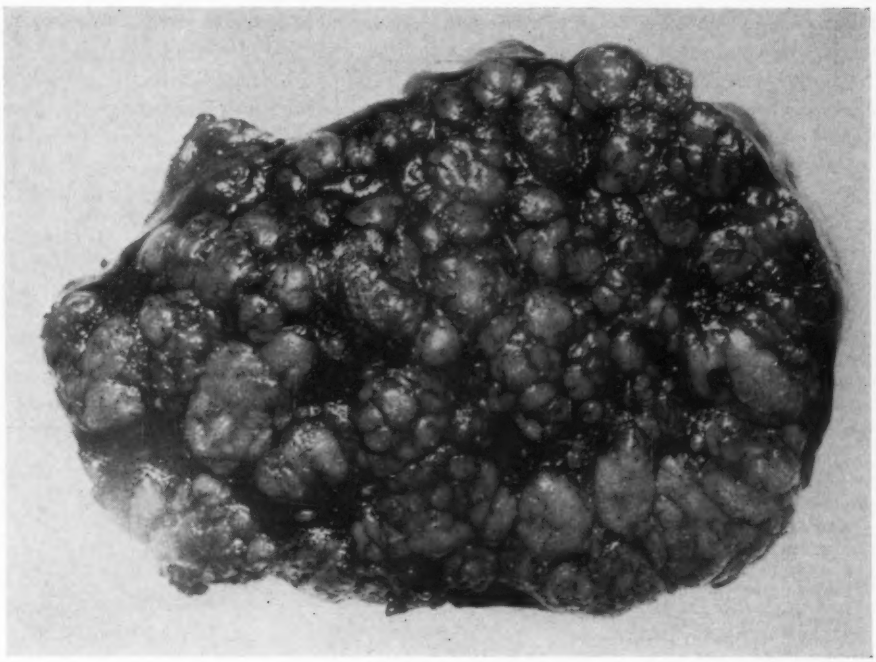

Fig. 1.—Spleen of a patient with lymphoma. Specific lymphoma tissue could be separated easily by enucleation.

2. *Extraction of Splenic Lipids.*—Splenic tissue was extracted in a 1/1 v.v. boiling mixture of acetone and absolute ethanol for 5 minutes. Extract was filtered immediately into a volumetric flask through Whatman No. 43 filter paper. After being cooled at room temperature, cold acetone-ethanol mixture was added to the extract, enough to bring the volume to 10 times the weight of original tissue used. Cholesterol and its fractions were determined in this final extract. This technique is not ideal for extraction of total lipids but yields cholesterol in its entirety.

3. *Determination of Cholesterol and Its Fractions.*—Total cholesterol was determined by a modification of the technique of Zlatkis and associates.[2] Aliquots of lipid extract

from splenic tissue (1 ml. of extract being equivalent to 0.1 Gm. wet tissue) were dried and redissolved in 3 ml. of acetic acid. Two milliliters of ferric chloride reagent were added. Further steps were as described by Zlatkis.[2]

Free cholesterol was determined with a technique which combines certain advantageous features of both Schönheimer's and Zlatkis' methods. Two milliliters of splenic extract were dried in a vacuum desiccator. Walls were washed with a small volume (0.2 to 0.3 ml.) of ethyl ether to collect the entire fraction to the bottom of the test tubes. Ether then was allowed to evaporate. Five drops of chloroform and 0.35 ml. of a solution of digitonin (1 per cent in 80 per cent ethanol) were added. Contents were stirred with a glass rod. Test tubes were then allowed to stand for 12 hours in atmosphere of ethanol under a glass bell. This step allowed complete precipitation of the digitonin-cholesteride complex without undue evaporation. Tubes were dried in a desiccator at 29″ vacuum; 2 ml. of ethyl ether then was added and the precipitate washed. After centrifugation, the supernatant ether was removed by suction and then evaporated in a water bath at 70° C. Precipitate was washed with 2 ml. of 60 per cent ethanol, 2 ml. of ethyl ether–acetone mixture, and 2 ml. of ethyl ether in succession, washing fluid being in each instance removed by suction and evaporation. Final precipitate was dissolved in 3 ml. of glacial acetic acid. Zlatkis' method then was followed. Values were calculated directly by reference to an experimental curve previously prepared, relating colorimetric readings to absolute cholesterol values. The curve was obtained as follows. Solutions were prepared containing known amounts of digitonin-cholesterol precipitates (pure cholesterol, Merck). Color was then developed with Zlatkis' reaction. Experimental colorimetric readings were then compared to the known cholesterol values.

Amount of esterified cholesterol was obtained by subtracting values of free from those of total cholesterol.

TABLE I. SPLEENS STUDIED IN THE PRESENT SERIES

	SURGICAL SPECIMENS	AUTOPSY SPECIMENS	TOTAL
Normal	2	2	4
Acute histiocytic leukemia	–	1	1
Erythroblastic leukemia	–	1	1
Chronic malignant reticuloendotheliosis	1	–	1
Lymphoma (with hemolytic anemia)	5	2	7
Myeloid metaplasia	1	–	1
Aplastic anemia	1	–	1
Hemoglobinopathy (Cooley's anemia)	1	–	1
Congenital spherocytosis	1	–	1
Acquired hemolytic anemia	3	–	3
Thrombocytopenic purpura (secondary)	2	–	2
Myocarditis	–	1	1
Chronic glomerulonephritis	–	1	1
Brain tumor	–	1	1
Cirrhosis of liver	–	1	1
Cerebral hemorrhage	–	1	1
	17	11	28

RESULTS

Values obtained for cholesterol and cholesterol esters in surgical and autopsy specimens were generally comparable. This was true for normal as well as for pathologic spleens. The finding eliminated a potentially important cause of error (Table II A, B, and C).

TABLE IIA. TOTAL CHOLESTEROL AND CHOLESTEROL ESTERS IN SPLENIC TISSUE (VALUES CALCULATED FOR WET TISSUE)

CASE	ORIGIN OF SPECIMEN	CHOLESTEROL (MILLIGRAMS PER CENT)			ESTERIFIED CHOLESTEROL (PER CENT)
		TOTAL	FREE	ESTERS	
		Normal			
1 H. C., ♂, 38	Surgical	340	90	250	73.6
2 E. A., ♀, 40	Surgical	340	75	265	78.0
23 S. S., ♂, 55	Autopsy	340	80	260	76.5
3 R. M., ♂, 48	Autopsy	350	85	265	75.7
Average		342.5	82.5	260	75.95
		Lymphoma			
4 M. F., ♀, 58	Surgical	670	490	180	26.8
5 J. M., ♂, 53	Surgical	420	165	255	60.7
6 C. B., ♀, 62	Surgical	530	290	240	45.3
7 A. Z., ♂, 27	Autopsy	450	180	270	60.0
8 G. B., ♀, 37	Autopsy	530	280	250	47.2
9 I. DiR., ♀, 19	Surgical*	580	208	372	64.1
10 E. G., ♂, 35	Surgical*	648	240	408	63.0
Average		546.9	264	282.1	52.4
		Hodgkin's Disease Tissue (separated from spleen)†			
6	Surgical	480	190	290	60.4
7	Autopsy	400	152	248	62.0
Average		440	171	269	61.2

*Spleens frozen in liquid nitrogen immediately upon collection.
†See Fig. 1.

TABLE IIB. TOTAL CHOLESTEROL AND CHOLESTEROL ESTERS IN PATHOLOGIC SPLENIC TISSUE: LEUKEMIAS (VALUES CALCULATED FOR WET TISSUE)

CASE	TYPE	ORIGIN OF SPECIMEN	CHOLESTEROL (MILLIGRAMS PER CENT)			ESTERIFIED CHOLESTEROL (PER CENT)
			TOTAL	FREE	ESTERS	
11 D. M., ♀, 39	Acute histiocytic	Autopsy	560	270	290	51.8
12 P. Mc., ♂, 53	Erythroblastic	Autopsy	550	230	320	58.2
13 E. O., ♀, 59	Chronic malignant reticuloendotheliosus	Surgical	439	202	237	53.8
Average			516.3	234	282.3	54.6

A. *Total and Free Cholesterol.*—Content of total and free cholesterol was high in abnormal splenic tissue irrespective of the pathologic picture. Thus, behavior of total and absolute free cholesterol was not diagnostic of any specific morbid condition.

B. *Cholesterol Esters.*—Absolute amount was normal or higher than normal in all diseases with involvement of the spleen, including lymphomas and leukemias. When, however, values of esterified cholesterol were compared to those of total cholesterol, it became apparent that there was a striking decrease of cholesterol esters in lymphoma and leukemia spleens and in isolated lymphoma tissue. These changes were, usually, less pronounced in other diseases with involvement of the spleen, although a picture indistinguishable from that found in lymphoma was found in a few instances (Cases 15, 17, 18, 20, 22).

TABLE IIC. TOTAL CHOLESTEROL AND CHOLESTEROL ESTERS IN SPLEENS OF PATIENTS WITH MISCELLANEOUS DISEASES (VALUES CALCULATED FOR WET TISSUE)

CASE	DIAGNOSIS	ORIGIN OF SPECIMEN	CHOLESTEROL (MILLIGRAMS PER CENT)			ESTERIFIED CHOLESTEROL (PER CENT)
			TOTAL	FREE	ESTERS	
26 M. S., ♀, 53	Hepatic cirrhosis	Surgical	290	90	200	68.2
21 M. C., ♂, 9 mo.	Cooley's anemia	Surgical*	330	100	230	69.7
14 E. C., ♀, 38	Idiopathic hemolytic anemia	Surgical*	310	85	225	72.5
27 T. M., ♂, 48	Brain tumor	Autopsy	430	95	335	78.0
24 S. G., ♀, 53	Chronic glomerulonephritis	Autopsy	360	71	289	80.3
25 F. K., ♂, 54	Myocarditis	Autopsy	580	160	420	72.4
17 M. M., ♀, 27	Scleroderma (ITP)	Surgical	620	280	340	54.8
18 E. G., ♀, 35	Sarcoidosis (ITP)	Surgical	525	280	245	46.7
15 E. D., ♀, 28	Congestive splenomegaly; hemolytic anemia	Surgical	615	275	340	55.3
16 J. L., ♂, 47	Hemolytic anemia (Brucellosis)	Surgical	495	202	293	59.2
22 M. T., ♂, 51	Myeloid metaplasia	Surgical	420	205	215	51.2
20 W. D., ♂, 22	Congenital spherocytosis	Surgical	460	215	245	53.3
19 R. Y., ♂, 32	Aplastic anemia	Surgical	405	120	275	68.0

*Spleens frozen in liquid nitrogen immediately upon collection.

C. *Comparison of Values of Total, Free, and Combined Cholesterol in Blood and in Tissue in Patients With Lymphoma.*—This was possible in many instances (Table III). Esterified cholesterol was relatively decreased in splenic

TABLE III. A COMPARISON OF CHOLESTEROL AND CHOLESTEROL ESTERS IN SPLENIC TISSUE* (SURGICAL SPECIMENS) AND SERUM IN PATIENTS WITH LYMPHOMA

CASE		CHOLESTEROL (MILLIGRAM PER CENT)			ESTERIFIED CHOLESTEROL (PER CENT)
		TOTAL	FREE	ESTERS	
4 M. F., ♀, 58	Serum	150	100	50	33.3
	Spleen	670	490	180	26.8
5 J. M., ♂, 53	Serum	162	95	67	41.4
	Spleen	420	165	255	60.7
6 C. B., ♀, 62	Serum	170	110	60	35.3
	Spleen	530	290	240	45.3
9† I. DiR, ♀, 19	Serum	145	95	50	34.5
	Spleen	580	208	372	64.1
10† E. G., ♂, 35	Serum	125	70	55	44.0
	Spleen	648	240	408	63.0
Average	Serum	150.4	94.0	56.4	37.7
	Spleen	569.6	278.6	291.0	51.98

*Values calculated for wet tissue.
†Tissue frozen immediately after collection.

tissue and, more so, in serum of lymphoma. To a smaller degree, this appeared also true of diseases with active involvement of the spleen (Table IV; Cases 15, 17, 18, 20, 22).

TABLE IV. A COMPARISON OF CHOLESTEROL AND CHOLESTEROL ESTERS IN SPLENIC TISSUE (SURGICAL SPECIMEN) AND SERUM IN PATIENTS WITH MISCELLANEOUS TYPES OF "HYPERSPLENISM"

CASE	DIAGNOSIS		CHOLESTEROL (MILLIGRAM PER CENT) TOTAL	FREE	ESTERS	ESTERIFIED CHOLESTEROL (PER CENT)
13	E. O., ♀, 59 Chronic malignant reticuloendotheliosis	Serum	160	70	90	56.3
		Spleen	439	202	237	54.0
19	R. Y., ♂, 32 Aplastic anemia	Serum	165	78	87	52.8
		Spleen	405	130	275	68.0
18	E. G., ♀, 35 Sarcoidosis (thrombocytopenic purpura)	Serum	138	70	68	49.2
		Spleen	525	280	245	46.7
17	M. M., ♀, 27 Scleroderma (thrombocytopenic purpura)	Serum	175	80	95	54.2
		Spleen	620	280	340	54.8
	Average	Serum	157.0	74.5	82.5	53.1
		Spleen	497.2	223.0	274.2	55.9

D. *Cholesterol Content in Arterial and Venous Splenic Blood.*—In 3 instances, satisfactory samples of arterial and venous blood could be obtained at surgery. In each instance, cholesterol level was slightly higher in the venous than in arterial blood. It is doubtful, however, whether these results are significant (Table V).

TABLE V. TOTAL CHOLESTEROL IN SPLENIC ARTERIAL AND VENOUS BLOOD IN 3 PATIENTS WITH LYMPHOMA

CASE	TOTAL CHOLESTEROL (MILLIGRAM PER CENT) ARTERIAL BLOOD	VENOUS BLOOD
5 J. M., ♂, 53	150	162
9 I. DiR., ♀, 19	130	145
10 E. G., ♂, 35	120	125

E. *Relationship of Cholesterol Content to Protein Structure in Splenic Tissue and Serum of a Patient With Lymphoma.*—It is generally considered that content and distribution of cholesterol and lipids in serum are related to a considerable extent to the content and structure of serum proteins. Studies on fasting patients with lymphoma indicated not only a defect of esterification of cholesterol but also considerable change in the distribution of cholesterol among the various protein fractions of serum. Electrophoretic distribution of protein fractions and of protein-bound cholesterol in the serum and splenic tissue therefore was determined (Table VI). Values given as normal represent the average of over 100 determinations with serum and of 5 studies with splenic tissue. In the case of lymphoma, results given represent the average of determinations on serum and splenic tissue of 5 patients. Techniques used were mostly elution procedures and they are being described in detail elsewhere.

TABLE VI. RELATIONSHIP OF CHOLESTEROL CONTENT AND DISTRIBUTION TO PROTEIN STRUCTURE IN TISSUE AND SERUM OF NORMALS AND PATIENTS WITH LYMPHOMA (SEE TEXT)*

			ALBUMIN + α_1-GLOBULIN		α_2-GLOBULIN		β-GLOBULIN		γ-GLOBULIN	
Normal serum	Proteins	(mg. %)	4,002	(58)	552	(8)	818	(11.6)	1,518	(22.4)
	Cholesterol	(mg. %)	39.89	(20.5)	31.2	(16.1)	56.98	(28.3)	71.87	(35.1)
Lymphoma serum	Proteins	(mg. %)	1,870	(31.8)	1,530	(20.5)	1,130	(19.1)	1,420	(24.0)
	Cholesterol	(mg. %)	58.3	(33.6)	31.7	(18.3)	24.2	(13.9)	59.3	(34.2)
Normal spleen	Proteins	(mg. %)	1,707	(47.6)	395	(11.0)	932	(26.0)	551	(15.4)
	Cholesterol	(mg. %)			30	(27.9)†	38	(35.1)	40	(37.0)
Lymphoma spleen	Proteins	(mg. %)	1,589	(27.5)	722	(12.5)	2,745	(47.5)	722	(12.5)
	Cholesterol	(mg. %)			70	(22.9)†	160	(51.5)	80	(25.6)

*Values in parentheses indicate percentage.
†α_1-+α_2-globulin fractions. No albumin fraction is usually present in splenic tissue.

Results in Table VI and Fig. 2 show close relationship between protein pattern of serum and tissues and electrophoretic migration of cholesterol. Lymphoma splenic tissue showed a striking increase of β-globulin as well as

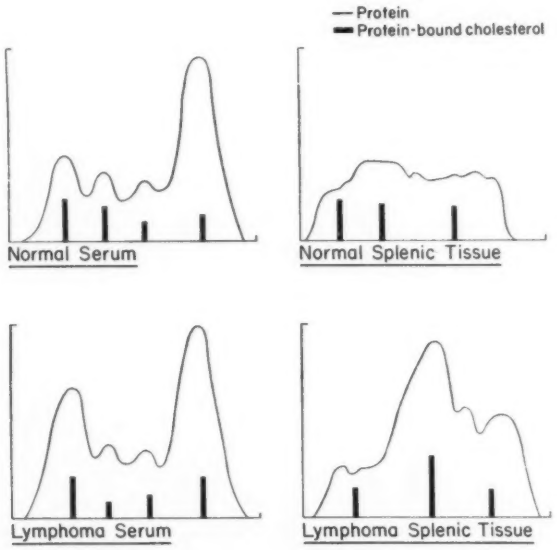

Fig. 2.—Electrophoretic distribution of protein fractions and protein-bound cholesterol in (*a*) normal serum, (*b*) lymphoma serum, (*c*) normal splenic tissue and (*d*) lymphoma splenic tissue. Note: (a) considerable increase of β-globulin in lymphoma tissue, (b) much higher concentration of protein-bound cholesterol in β-globulin fraction in lymphoma splenic tissue, and (c) the apparent relative inability of serum β-globulin to carry cholesterol in lymphoma patients (see text).

a high content of cholesterol. Serum of patients with lymphoma, on the contrary, showed normal β-globulin but low cholesterol content. It is possible then that binding by β-globulin in tissue may have contributed to the high tissue content of cholesterol and, consequently, low serum cholesterol content in serum of patients with lymphoma.

DISCUSSION

A great deal of attention has been given to changes in protein and lipid content in pathologic sera. Among the lipids, the behavior of cholesterol has been extensively studied. Changes in serum cholesterol and in its esterification

are recognized as an important index of disease. Decrease of total level and impaired esterification of cholesterol have been described in parenchymal liver disease, malignancies, severe anemia, and lymphomas. The significance of these changes and, especially, their mechanism remains obscure. Particularly, it is unknown whether changes in serum are a reflection of similar ones within tissues. A review of the literature shows that the entire subject of the relationship of lipid constituents of tissues to those of serum has received little attention. Billing and associates,[3] among others, recently have observed a satisfactory relationship of values of total lipids in liver tissue as compared to serum in cirrhosis of the liver. There are, however, no systematic studies on cholesterol content and partition in serum as compared to tissues in diseases where serum cholesterol changes appear important.

Fig. 3 summarizes our results and is offered as a guide to a brief discussion. Three findings of major interest resulted from our studies: (a) *significantly elevated total cholesterol in diseased tissue:* A higher content of total cholesterol was found in pathologic spleens, whether the spleen was or was not the

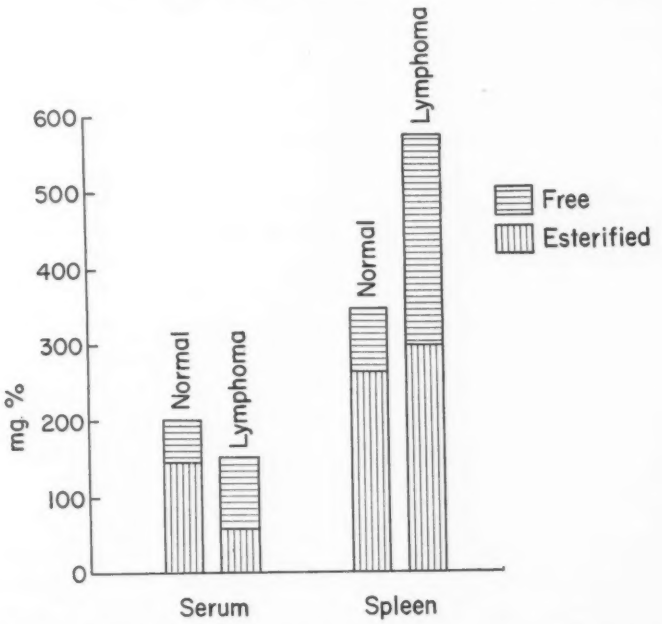

Fig. 3.—Total, free cholesterol and cholesterol esters in serum and in splenic tissue of healthy individuals and patients with lymphoma. Note: (a) Lymphoma serum: total and esterified cholesterol are decreased, (b) Lymphoma splenic tissue: total cholesterol is absolutely increased but esterified cholesterol is relatively decreased.

Total cholesterol is higher than normal in splenic tissue and lower than normal in serum of patients with lymphoma. Esterification of cholesterol, however, is subnormal in both instances.

organ primarily involved by the fundamental disease. It would appear that accumulation of cholesterol in the spleen (perhaps other organs) is a nonspecific indication of disease, irrespective of its nature and of the organ primarily involved by it; (b) *defect of esterification of cholesterol, especially evident in lymphoma:* Although the absolute amount of cholesterol was usually increased, the percentage of esterified cholesterol was much lower in abnormal

than in normal spleens.* Poor cholesterol esterification was most striking in the lymphoma and leukemia spleens. This finding remains unexplained and is under investigation at present; (c) *striking dissociation between high cholesterol content in splenic tissue and low content in serum in patients with lymphoma, although the defect of esterification could be observed in both:* A partial answer to this finding was provided by electrophoretic studies, as previously discussed. These indicated a relative increase of β-globulin content in the lymphoma spleen as compared to the normal spleen, far in excess of a similar trend observed in serum. Since a considerable amount of cholesterol is bound to β-globulin, the high β-globulin in tissues may prevent normal delivery of cholesterol into the blood.

Another interesting observation resulted from the analysis of data in Table VI. Albumin fraction is usually low in the serum of patients with lymphoma. Still, the albumin in lymphoma serum contained at least one-third of bound cholesterol as compared to one-fifth for normal serum. Also, the β-globulin fraction was higher in lymphoma serum than in normal serum; still the β-globulin of lymphoma serum contained less than one-half of the cholesterol found in the same fraction of normal serum. These figures suggest poor binding capacity of β-globulin for cholesterol in the serum of patients with lymphoma. They may, in addition, indicate an abnormality of structure of this protein. Such a conclusion is also suggested by other findings obtained in this laboratory.

The significance of these studies cannot be properly assessed at the present time. They indicate an abnormality of partition and of transport of cholesterol in many disease states and, particularly, in lymphoma. Whether these changes are identifiable with any specific condition or whether they bear any relationship to the pathogenesis of the disease involved will be established only by further work. Our results, however, among the first to indicate a well-defined metabolic abnormality in lymphoma, further stress the potential importance of the biochemical study of tissues in the appreciation of the molecular disturbances which may be at the basis of the pathogenesis of some disease states.

SUMMARY

1. Systematic studies of tissue (spleen) and serum cholesterol in various pathologic states indicate that: (a) tissue content of total cholesterol is elevated in pathologic states, especially those involving the spleen. Serum, at the same time, shows low total cholesterol level. Thus, accumulation of cholesterol in the spleen seems typical of disease states, especially those involving the spleen; (b) cholesterol esters are relatively decreased in serum and tissues alike. Thus, percentage of esterified cholesterol is significantly low.

2. The defect of esterification of cholesterol remain unexplained at the present time. On the other hand, accumulation of cholesterol in tissue as compared to serum indicates an abnormality of cholesterol transport. It may be

*There were only two exceptions, Cases 14 and 21. It may be noted, however, that spleen No. 21 belonged to a 9-month-old baby. The influence of age on esterification of tissue and serum cholesterol has not been studied in man. It is known, however, that organs of newly hatched chickens have high cholesterol esters content.[4]

due, at least in part and in cases with lymphoma, to the high content of β-globulin in the pathologic splenic (perhaps other) tissue as compared to normal spleen.

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THE EFFECT OF LIVER DISEASE ON SERUM VITAMIN B_{12} CONCENTRATIONS

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INTRODUCTION

SINCE the isolation and crystallization of vitamin B_{12} in 1948,[1] there has been increasing interest in the fundamental metabolic action of this substance. Although most studies have been concerned with its role in red cell maturation, it has become increasingly evident that this vitamin has other important functions. Experimentally, vitamin B_{12} appears to be important in the intermediate metabolism of proteins, particularly in the synthesis of nucleoproteins.[2-4] Also, it seems to have a role in the metabolism of both carbohydrates and fats.[5-7] It has been reported to have a protective action against liver necrosis in experimental carbon tetrachloride intoxication,[8, 9] and it also exerts a lipotropic effect in animals fed choline- or methionine-deficient diets.[10, 11] Experiments with radioactive vitamin B_{12} have shown that the liver is the major storage depot of this substance in the human body.[12, 13]

The extremely minute amounts of vitamin B_{12} present in body fluids and the relatively small amounts of these fluids which can be made available to the laboratory have precluded the measurement of vitamin B_{12} by the usual chemical methods and have necessitated the employment of various microbioassay techniques. The methods of assay most generally used depend on the fact that certain lactobacilli require this vitamin as an essential growth factor. However, assays with these organisms are not entirely specific and have a rather low sensitivity which makes them unsatisfactory for detecting the low concentrations of vitamin B_{12} in the body fluids. In 1952, Ross[14] first described the microbiologic determination of vitamin B_{12} in body fluids using *Euglena gracilis*, var. *bacillarus*. This method is based on Hutner's work[15] which showed that Euglena exhibit a quantitative growth response to vitamin B_{12}. By this technique, it is possible to measure vitamin B_{12} activity in body fluids in concentrations as low as 100 $\mu\mu$g/ml., and also to measure separately both the free and combined forms which are found in serum. In the present study this method has been employed to clarify the metabolism of vitamin B_{12} in patients with liver disease. Preliminary reports of certain phases of this study have been published.[16, 17]

MATERIALS AND METHODS

Thirty-six patients with various types of liver disease were studied over an 18-month period, many of the patients being followed continuously during this interval. Only patients with clear-cut objective evidence of liver disease were included; otherwise the patients were unselected and chosen entirely at random. Eleven patients gave a definite

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history of excessive alcoholic intake and were classified as having "alcoholic" or "Laennec's" cirrhosis. Ten of these patients had the clinical diagnosis confirmed by a pathologic examination of liver tissue. Ten patients were classified as postnecrotic cirrhosis. Many of the patients in this group gave a history of antecedent jaundice in years past, and none gave a history of having used excessive amounts of alcohol. Nine of these patients had the clinical diagnosis confirmed by liver biopsy. Six patients were studied during the icteric and convalescent stage of acute viral hepatitis. Five patients had biliary cirrhosis and all the patients in this group had the diagnosis verified by liver biopsy. Four patients were studied during periods of hepatic coma secondary to chronic liver disease and all of this group subsequently expired.

Serial determinations of serum vitamin B_{12} concentrations were made using *Euglena gracilis*, var. *bacillarus* as the test organism. The method used was that described by Ross.[14] Each sample of serum was assayed in at least 2 dilutions before and after heating at 100° C., and each dilution was run in quadruplicate. The values obtained after heating the serum are considerably higher than those obtained without heating and are designated as "total" vitamin B_{12}. The values determined in unheated serum are referred to as "free" or "uncombined" vitamin B_{12}. The majority of the serum vitamin B_{12} is tightly bound to what is presumably a protein and is available to the Euglena only after heating the serum. This fraction, the "bound" or "combined" vitamin B_{12}, is determined indirectly by subtracting the value obtained for the free vitamin B_{12} from the total vitamin B_{12}.

Serial liver function tests were performed throughout the period of study; these included quantitative van den Bergh reaction, cephalin cholesterol flocculation, thymol flocculation and turbidity, zinc sulfate turbidity, ammonium sulfate turbidity, alkaline phosphatase, serum albumin and globulin, serum cholinesterase, and Bromsulphalein retention. The methods used for these liver function tests have been previously described.[18]

Eleven healthy adults, ranging between 21 and 33 years of age, were used as controls. These subjects had normal liver function tests and were without signs or symptoms of hepatic disorders. Their sera were assayed in a manner identical with that used in the bioassay of the patients' sera.

Twenty-four-hour urine collections were bioassayed for vitamin B_{12} concentration in 14 of the patients with liver disease and in 10 of the normal controls.

The vitamin B_{12} binding capacity of serum was studied in vitro in a limited number of cases. Aliquots of serum samples from these patients were first assayed for both the free and the bound form of vitamin B_{12} and then duplicate samples were mixed and incubated with either 500 or 1,000 $\mu\mu$g of crystalline vitamin B_{12} in the manner described by Pitney and associates.[19] The incubated serum was then reassayed for vitamin B_{12} content. In vivo tolerance studies were also conducted in a limited number of patients. After urine and serum control values were obtained, the patients were given 30 μg of crystalline vitamin B_{12} intramuscularly. The serum was then assayed for vitamin B_{12} content at 4, 8, 24, 48, and 72 hours and again at the end of one week. Simultaneous 24-hour urine collections were bioassayed for vitamin B_{12} concentration.

RESULTS

Serum Vitamin B_{12} Concentrations.—The range and mean values obtained in the normal group are presented in Table I. These subjects were studied with multiple assays. We found, as did Ross, that although there is a considerable range among normal subjects, for any one given individual, the

TABLE I. SERUM VITAMIN B_{12} CONCENTRATIONS IN ELEVEN NORMAL SUBJECTS

	SERUM VITAMIN B_{12} CONCENTRATIONS ($\mu\mu$g/ML.)	
	FREE	COMBINED
Range	24-147	154-428
Mean	72	248
S.e. of Mean	±13	±23

serum vitamin B_{12} concentrations do not alter significantly from month to month, and the slight changes that were found occurred mainly in the uncombined fraction. The mean value for the total serum vitamin B_{12} in this group was 320 $\mu\mu$g/ml., which compares favorably with a mean value of 358 $\mu\mu$g/ml. as reported by Ross for 65 normal subjects in the same age group.[14, 20]

One hundred thirteen serial bioassays were performed on the sera from the 36 patients with various hepatic disorders. Because of the constant change in the severity of the liver disease in the majority of these patients, it is impossible to analyze the entire results of the serial tests over the 18-month

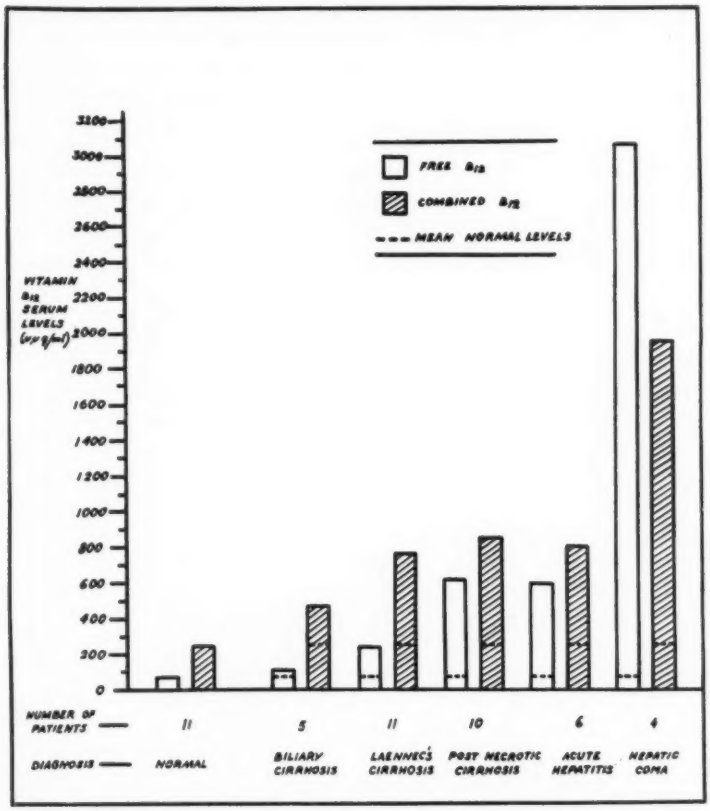

Fig. 1.—Combined and free vitamin B_{12} serum levels in patients with liver disease compared with the values obtained in normal subjects.

period of study and derive any so-called "average" or "mean" for each individual or for any one group. Instead, the highest total serum vitamin B_{12} concentration for each individual during the period of observation was selected and the free and combined serum levels were analyzed. The range and mean values thus obtained are presented in Table II. These mean values are graphically compared with the normal mean values in Fig. 1. It can be seen that all of the serum vitamin B_{12} levels in patients with liver disease, with the exception of those with biliary cirrhosis, are significantly elevated over the

normal control values. Although there appeared to be wide variation of the individual determinations within their groups, when the data from any one individual were studied, it was found that the most abnormal levels occurred at a time when the patient was the most acutely ill. As the patients clinically improved, the serum vitamin B_{12} concentrations tended to return toward the normal range, and conversely, when those patients who were in hepatic coma became moribund, the serum vitamin B_{12} levels became more abnormal. Thus, serum vitamin B_{12} concentrations roughly paralleled the functional state of the liver as judged by clinical and laboratory findings; however, there was no apparent correlation with any one specific laboratory test. It can also be seen in Table II that those patients having acute inflammation and necrosis at the time of study, i.e., those with acute viral hepatitis, postnecrotic cirrhosis, and those in liver coma, had a higher proportion of the free form than did those patients with Laennec's cirrhosis.

TABLE II. FREE AND COMBINED SERUM VITAMIN B_{12} CONCENTRATION IN VARIOUS TYPES OF LIVER DISEASE

DIAGNOSIS	NUMBER OF PATIENTS	AGE	SERUM VITAMIN B_{12} CONCENTRATIONS ($\mu\mu$g/ML.)*			
			FREE		COMBINED	
			RANGE	MEAN	RANGE	MEAN
Acute hepatitis	6	18-30	261-1040	589	80-1563	808
Laennec's cirrhosis	11	29-52	58- 501	232	317-1306	759
Postnecrotic cirrhosis	10	12-52	237-1360	607	516-1500	856
Biliary cirrhosis	5	40-55	26- 276	111	203- 928	468
Hepatic coma	4	32-84	1600-6368	3072	512-2944	1964

*The range and the mean values are derived from the most abnormal levels for each individual patient.

TABLE III. TWENTY-FOUR-HOUR URINE VITAMIN B_{12} CONCENTRATIONS (μg/24 HOURS)

DIAGNOSIS	NUMBER OF PATIENTS	URINE VITAMIN B_{12} CONCENTRATION (μg/24 HOURS)	
		RANGE	MEAN
Normal	10	0.08-0.23	0.11
Laennec's cirrhosis	5	0.34-6.1	1.82
Postnecrotic cirrhosis	5	0.34-5.8	3.21
Acute hepatitis	4	0.38-3.38	1.35

Urine Vitamin B_{12} Concentrations.—Twenty-four-hour urine collections were assayed for vitamin B_{12} concentrations in 10 normal subjects and in 5 patients with Laennec's cirrhosis, 5 patients with postnecrotic cirrhosis, and 4 patients with acute hepatitis. All of the vitamin B_{12} appeared to be in an uncombined form; no additional growth was obtained after the urine had been heated. The range and mean values thus obtained are shown in Table III. It can be seen that, although there is a wide spread of the range between individual patients, all of the patients with liver disease excreted comparatively large amounts of vitamin B_{12} during each 24-hour period studied.

Serum Binding Capacity.—Studies were undertaken to measure the capacity of serum to combine with vitamin B_{12} in vitro. Serum samples from both normal subjects and patients were incubated with either 500 $\mu\mu$g or 1,000

$\mu\mu$g of crystalline vitamin B_{12}. In general, it was found that if adequate amounts of free vitamin B_{12} were present in the serum, very little additional vitamin B_{12} could be bound regardless of the amount of crystalline vitamin B_{12} added. The behavior of the sera of both the normal subjects and the patients with the liver disease was similar, the main difference being in the amount of bound vitamin B_{12} initially present in the control values (Fig. 2). In vivo tolerance studies were also made in a limited number of normal subjects and patients. After control values for both serum and urine were obtained, the patients were given 30 μg crystalline vitamin B_{12} intramuscularly. The serum was then assayed for the vitamin B_{12} content at 4, 8, 24, 48, and

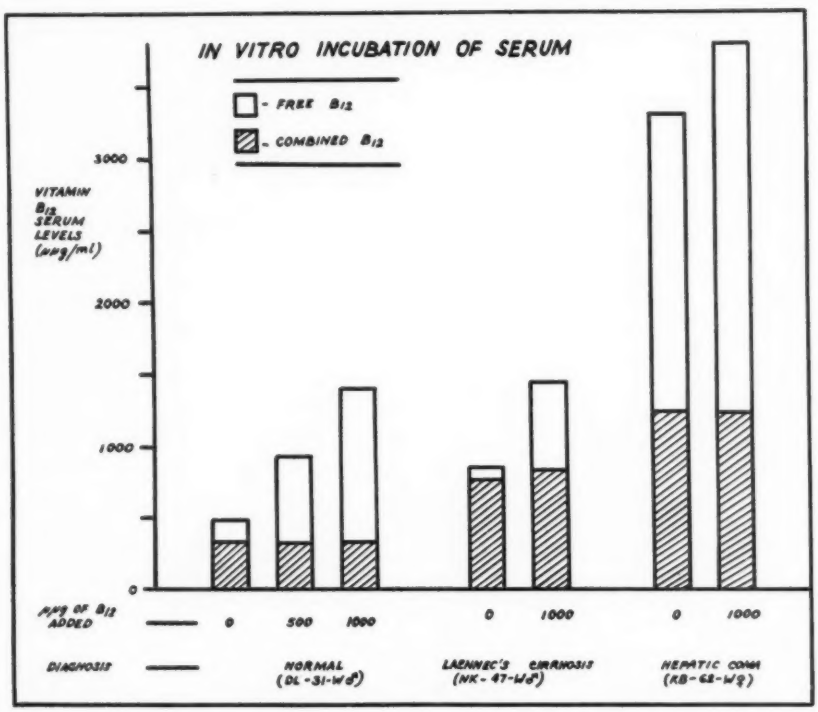

Fig. 2.—Combined and free serum vitamin B_{12} levels in one normal subject and in 2 patients with liver disease before and after in vitro incubation of the sera with pure crystalline vitamin B_{12}.

72 hours and again at the end of one week after the injection. Simultaneously, collected 24-hour urine specimens were also assayed for vitamin B_{12} concentrations (Figs. 3 and 4). It can be seen that there was an appreciable rise in the total serum vitamin B_{12} concentrations after the injection of the vitamin B_{12} which reached the maximum level at the end of 4 hours. Again, as in the in vitro studies, very little additional vitamin B_{12} was bound, most of the increase in the serum concentrations being in the free fraction. By the end of 24 hours the serum levels had returned to the control range; however, only a small percentage of the injected vitamin B_{12} could be accounted for in either the serum elevation or in the urine. It must be assumed that the remainder of the injected dose was either metabolized or stored in the tissues.

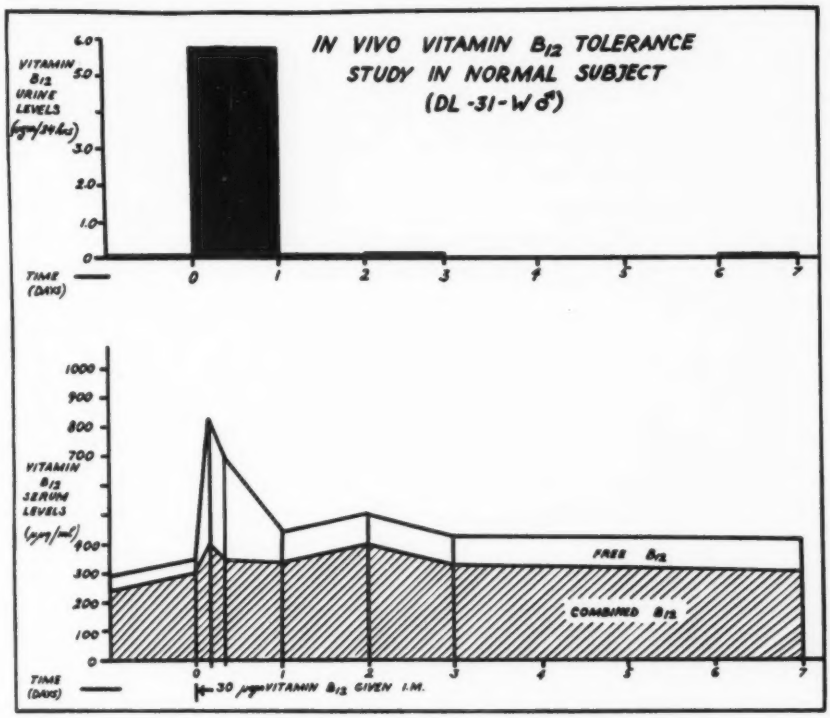

Fig. 3.—In vivo vitamin B_{12} tolerance study in a normal subject.

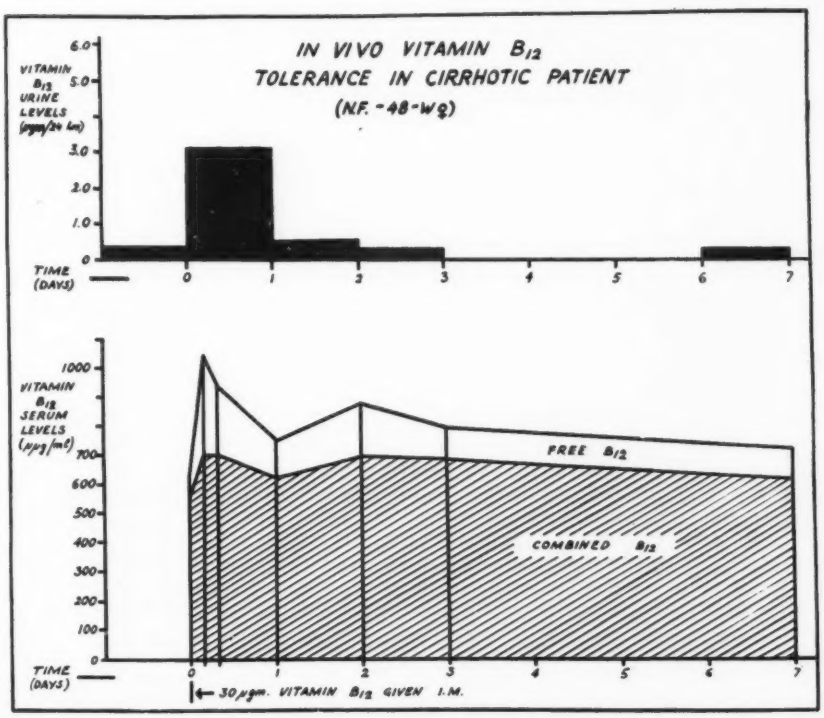

Fig. 4.—In vivo vitamin B_{12} tolerance study in a patient with alcoholic cirrhosis.

CASE REPORTS

In order to demonstrate the individual variations of serum vitamin B_{12} concentrations in relation to the course of the patient's illness, 3 representative cases have been selected for individual presentation. The serum vitamin B_{12} levels and selected liver function tests are shown graphically in the figures. The vertical lines represent individual observations. The horizontal lines represent the upper limits of normal (i.e., 2 standard deviations of the mean) for the liver function tests, while in the graphs of the serum and urine vitamin B_{12} levels they represent the upper limits of the normal range.

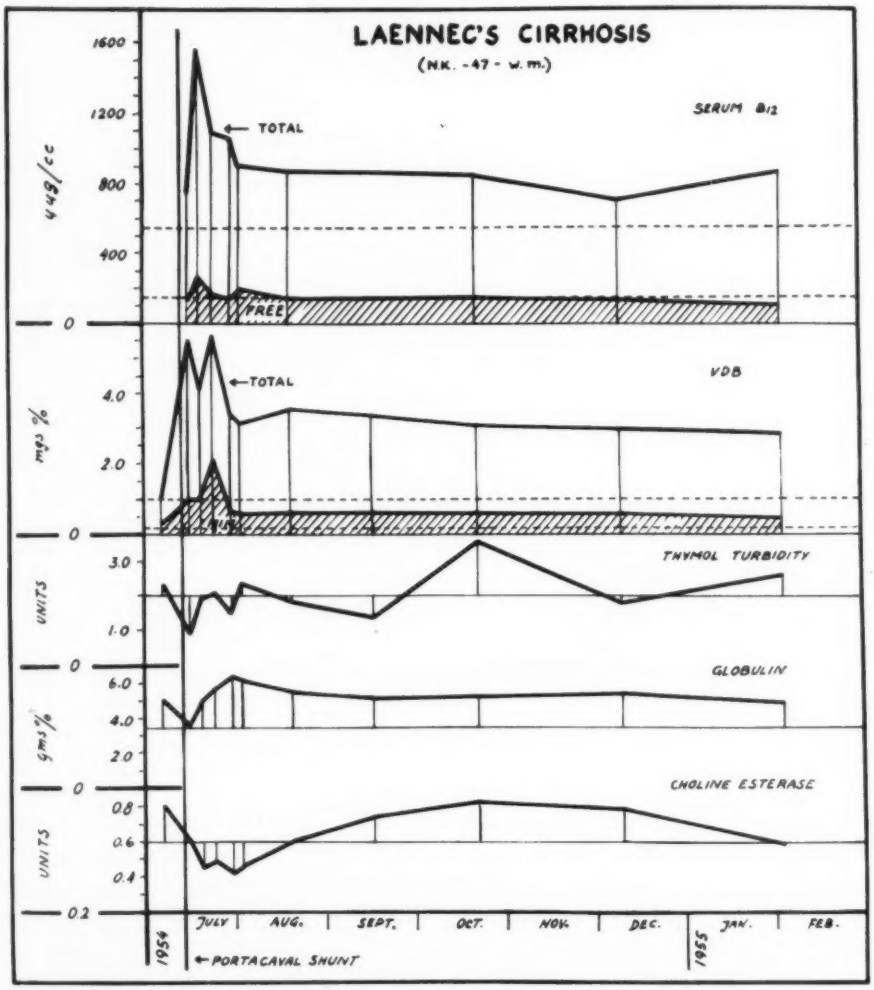

Fig. 5.—Course of a patient with alcoholic cirrhosis. Horizontal lines indicate normal values.

CASE 1 (N. K., Fig. 5).—This 47-year-old white man was admitted to St. Luke's Hospital on July 1, 1954. He had been in good health until October, 1953, at which time he passed tarry stools. After 3 weeks of observation at another hospital, the patient was

surgically explored. The only positive finding was a grossly cirrhotic liver. In April, 1954, the patient had hematemesis which stopped spontaneously. Over the past twenty years the patient drank 10 to 12 bottles of beer and 6 to 15 ounces of whisky per day. There was no past history of jaundice. Physical examination revealed an obese white man. The patient had marked palmar erythema and spider nevi. The liver was palpated 8 cm. below the right costal margin in the midline. The spleen was not felt. There was 2+ pitting edema over the ankles. In addition to the laboratory data shown in Fig. 5, the patient had a Bromsulphalein retention of 32.1 per cent at 45 minutes, an alkaline phosphatase of 11.2 Bodansky units, and a red count of 3,540,000. Esophagoscopy demonstrated large varices. Gastrointestinal x-rays were normal. On July 13, 1954, a portacaval shunt and a liver biopsy were performed. The spleen was estimated to be at least twice normal size. The liver biopsy showed a typical advanced portal cirrhosis. The patient has been followed continuously since the day of surgery. Esophagoscopy in June, 1955, showed no evidence of the varices.

Comment.—This patient represents a typical advanced Laennec's cirrhosis. The total serum vitamin B_{12} concentration, representing mainly the combined fraction, remained persistently elevated. During the week following surgery there was a transient elevation of the serum vitamin B_{12}.

CASE 2 (S. C., Fig. 6).—This 26-year-old white man was admitted to St. Luke's Hospital July 1, 1954. Four days prior to admission the patient developed a sudden chill with profuse sweating. During the next 3 days he had occipital headaches, extreme malaise, and a low-grade fever. Three days prior to admission he began to pass dark brown urine. He complained of slight pain in the right upper quadrant when walking. Physical examination revealed a well-developed white man with obvious icteric sclera. There were palpable cervical, axillary, and inguinal lymph nodes and the spleen was felt to be slightly enlarged. The liver was enlarged to 4 cm. below the right costal margin and was slightly tender. The patient was treated with bed rest and placed on a high carbohydrate, high calorie diet. The laboratory data are shown in Fig. 6. Heterophile agglutination on July 3, 1954, was positive in a dilution of 1:256. The patient was discharged on July 12, 1954, to his home for convalescence.

Comment.—Although this patient was felt to have infectious mononucleosis with moderately severe hepatitis, the pattern of his serum B_{12} concentration was typical of that found in most of the other cases of acute viral hepatitis. There is a marked rise in the total serum vitamin B_{12} in the early icteric phase of the disease, practically all of which is in the free fraction. During the time when the serum B_{12} is elevated, there is a considerable elevation in the urinary excretion of vitamin B_{12}. The serum vitamin B_{12} levels promptly return to normal as the patients recover.

CASE 3 (T. Z., Fig. 7).—This 38-year-old white woman was admitted to St. Luke's Hospital Dec. 2, 1953. For many years the patient had worked in a factory which made certain types of plastics and was constantly exposed to potentially hepatotoxic vapors. She felt relatively well until 6 months prior to admission at which time she had a hysterectomy done in another hospital. The patient had a stormy postoperative course and never regained her normal sense of well-being. No note was made of the condition of the liver at that time. There was no blood or plasma received during this hospitalization. A few weeks prior to admission to this hospital the patient had an episode of abdominal cramp-like pains and nausea. It was at this time that she noted that her stools were becoming light and that her urine was becoming dark brown. Shortly after this she noted that her sclera and skin were becoming jaundiced. The patient had never been jaundiced prior to the current illness and had never taken more than a moderate amount

of alcoholic beverages. Physical examination revealed a well-developed white woman who was deeply jaundiced. The liver was felt 5 cm. below the right costal margin and was slightly tender. She was treated as an acute hepatitis, but because of the chronicity of the course, a liver biopsy was done. This was performed on Dec. 29, 1953, and showed rather marked cirrhotic changes which were interpreted as being compatible with the postnecrotic type of cirrhosis. The patient has been followed continuously since this time.

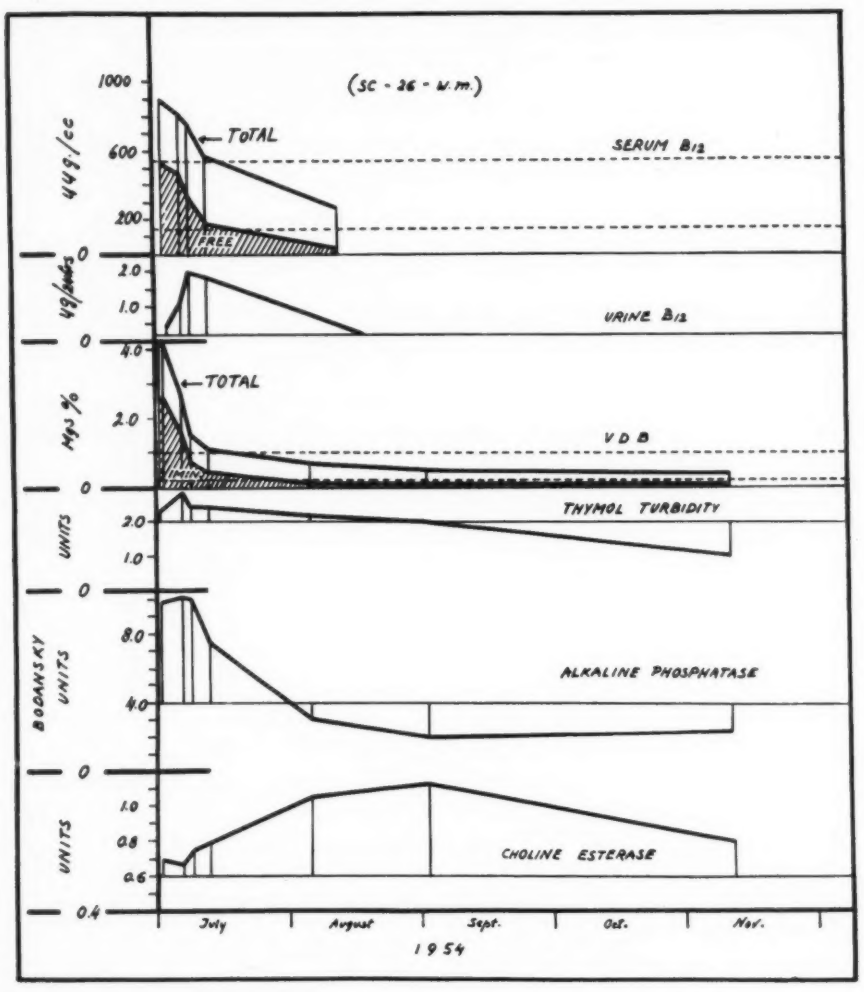

Fig. 6.—Course of a patient with infectious mononucleosis and moderately severe hepatitis. Horizontal lines indicate normal values.

ACTH was given with dramatic results as indicated in Fig. 7, both in the sense of well-being and in the liver function tests. The Bromsulphalein retention test, which was 43.9 per cent at 45 minutes, dropped to 3.8 per cent retention in 45 minutes in January, 1955.

Comment.—This patient was felt to have chronic active liver disease with a marked amount of inflammation at the onset of her illness. The serum vitamin B_{12} showed a marked rise in the free fraction similar to that found in patients with acute viral hepatitis. The pattern of the serum vitamin B_{12} levels

in this patient was found in other patients with chronic active liver disease; as the disease became more chronic and the inflammatory phase became less prominent, the serum vitamin B_{12} levels began to resemble those found in Laennec's cirrhosis. It is of interest to note the effect of ACTH in this patient. The serum vitamin B_{12} levels returned to near normal as did many of the liver function tests while the patient was receiving this drug.

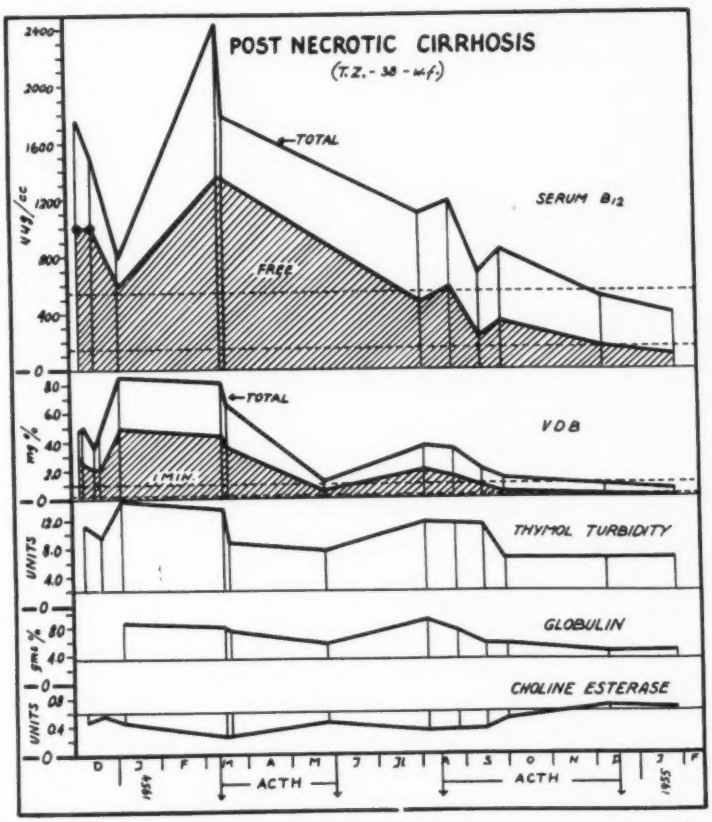

Fig. 7.—Course of a patient with postnecrotic cirrhosis treated with ACTH. Horizontal lines indicate normal values.

DISCUSSION

It is obvious from our study that there is a marked increase in the growth-supporting activity for *Euglena gracilis* in the sera of many patients with liver disease. The question has to be raised, however, whether this material is, in fact, vitamin B_{12}. Such a conclusion is, of course, given strong support by the findings of others[14, 15, 21] who report a high degree of specificity of vitamin B_{12} for *Euglena gracilis*. The few naturally occurring substances which are capable of substituting for vitamin B_{12} have never been identified in human serum. Furthermore, our data indicate that the absolute rise in the level of growth-promoting substance in the serum following the injection of a standard

unit of crystalline vitamin B_{12} is related to the control level as would be expected if we were dealing with the same substance. One other possibility, however, must be considered; namely, that some or all of the excessive growth-promoting factor present in liver disease may be an abnormal type of vitamin B_{12}.

Although we were able to demonstrate a small amount of uncombined vitamin B_{12} in all of our normal subjects, most of the vitamin B_{12} found in serum is combined with a substance which presumably is a protein. After electrophoresis of serum, the bound B_{12} is located mainly in the alpha-globulin fraction.[19] All of the patients with liver disease had significantly increased amounts of total vitamin B_{12} in their sera, except for a few of those with biliary cirrhosis and a few with Laennec's cirrhosis. It is significant to note that these normal values were found only in well-stabilized patients who appeared to have no progression of the disease at the time of study. The patients with acute hepatitis had large amounts of free vitamin B_{12} present in their serum but had very little increase in the amount of combined vitamin B_{12}. Patients with more chronic liver disease had a rather marked increase in the combined fraction and also an increase in the free form. This was most marked in patients in liver coma where the total vitamin B_{12} values were some 15 times greater than the normal levels.

It is generally agreed that the capacity of normal serum to bind vitamin B_{12} is limited.[19, 22, 23] Our studies support this conclusion. The binding capacity of the serum from patients with Laennec's cirrhosis, although increased over the normal levels, also appears to be limited, and in our patients it was not possible to significantly increase the amount of bound vitamin B_{12} by either incubating the serum with the vitamin or by giving injections of crystalline vitamin B_{12} intramuscularly. The amount of vitamin B_{12} found in the urine appears to be closely related to the amount of circulating free vitamin B_{12}, both in the normal subjects and in patients with liver disease.

The significance of these findings is uncertain. Others have also found high serum vitamin B_{12} concentrations in patients with acute and chronic liver disease.[23, 24] Increase in vitamin B_{12} serum levels has been found in patients with various hematological diseases, i.e., chronic and acute myelocytic leukemia, acute monocytic leukemia, polycythemia rubrum vera, myelosclerosis, and chronic nonleukemic leukocytosis, and the significance of the elevation in these conditions has not been definitely established.[20, 25-27] It is known that the liver is the major storage depot in the body for vitamin B_{12},[12, 13] and the vitamin B_{12} appears to exist in the liver in a loosely-bound protein complex which has an electrophoretic mobility similar to that of serum beta-globulin.[28] It is possible that the high serum concentrations in liver disease are a result of the release of both free and bound vitamin B_{12} by the breakdown and necrosis of abnormal numbers of liver cells. This would account for the marked increase in the free serum vitamin B_{12} in the patients with acute hepatitis and in those with liver coma. There also appears to be an increase in the binding substance in many of our patients, particularly those with chronic liver disease. It has long been known that patients with cirrhosis produce an

abnormal amount of globulins. As it has been shown that the binding material is presumably an alpha-globulin,[19] it would seem reasonable to assume that this could account for the increase in binding substance found in these patients.

It would seem difficult to correlate our findings of elevated serum vitamin B_{12} levels with the reports in the clinical literature of the beneficial effects of B_{12} therapy in patients with liver disease.[29, 30] The value of drugs in influencing the clinical course of liver disease is very difficult to assess and it is possible that the true benefits of this therapy are not as great as the authors thought. On the other hand, the large amount of growth-supporting substance found in the sera of our patients might be physiologically inactive. This could conceivably be due to either an abnormal type of vitamin B_{12} or due to an abnormal binding in the serum. If this were the case, one might expect a deficiency of physiologically active vitamin B_{12} and the body tissues to be relatively unsaturated. This can be settled only by further studies.

SUMMARY

1. Serum vitamin B_{12} concentrations were determined in 36 patients with liver disease; 11 patients had alcoholic cirrhosis, 10 patients had postnecrotic cirrhosis, 6 patients had acute viral hepatitis, 5 patients had biliary cirrhosis, and 4 patients were studied during liver coma.

2. All of the patients had total serum vitamin B_{12} levels markedly elevated over the normal control values with the exception of those with biliary cirrhosis.

3. Patients with acute inflammation and necrosis had relatively high levels of free vitamin B_{12} while those with chronic liver disease had elevated bound vitamin B_{12}.

4. Twenty-four-hour urine collections were assayed for vitamin B_{12} content in 14 patients with liver disease and in 10 normal controls. All of the patients with liver disease excreted comparatively large amounts of vitamin B_{12}.

5. In vitro incubation studies and in vivo tolerance tests were done in both normal subjects and patients with liver disease in an effort to study the binding capacity of the serum. In most of the cases the serum binding capacity appeared to be saturated when studied by either of these techniques.

6. The possible significance of these results is discussed.

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RENAL CONCENTRATING MECHANISM IN SICKLE-CELL ANEMIA

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INTRODUCTION

IT IS known that in patients with sickle-cell anemia the concentrating power of the kidneys during hydropenia is less than in normal subjects.[1-4] The defect in children is not accompanied by a reduction in filtration rate or effective renal plasma flow,[5, 6] though these functions may be decreased in adults, presumably in consequence of thrombus formation and loss of functioning tissue.[7] The defect in concentrating power is not related to deficient activity of the osmoreceptors, since some measure of antidiuresis is elicited by hypertonic saline. Moreover, concentrating activity is not enhanced by the administration of Pitressin.[8] These observations indicate that the defect is referable to the concentrating mechanism in the kidney. However, it is not attributable to anemia per se since a similar defect has been reported in patients with sickle-cell trait, but normal hemoglobin levels and normal concentrating power have been reported in patients with other types of hemolytic anemia.[9]

Recent studies by Keitel, Thompson, and Itano[10] indicate that the defect can be remedied completely in infants (1 to 3 years of age) if their erythrocytes are exchanged for normal cells and a normal hemoglobin level is maintained for several weeks. As the infused red cells disappear and the hemoglobin level decreases, the defect reappears. Thus it seems that the defect may be related to some unique feature of sickle-cell disease rather than to anemia itself.

The present study is directed toward a more quantitative description of the renal concentrating defect in this disease, approaching the problem in terms of the recent studies of Zak, Brun, and Smith[11] on the nature of the concentrating operation in normal subjects.

The volume of solute-free water ($T^c_{H_2O}$) abstracted from an isosmotic tubular urine (or glomerular filtrate) in concentrating the urine is calculated as the difference between the osmolar clearance (C_{OSM} or $U_{OSM}V/P_{OSM}$) and the urine flow (V):

$$T^c_{H_2O} = \frac{U_{OSM}V}{P_{OSM}} - V \qquad \text{I.}$$

Zak, Brun, and Smith[11] have shown that during osmotic diuresis in normal subjects $T^c_{H_2O}$ becomes approximately constant and they designate the limit-

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TABLE I. OSMOTIC CONCENTRATING OPERATIONS IN PATIENTS WITH ANEMIA

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
PATIENT	AGE	SEX	S.A. (SQ.M.)	HB. (GM./100 ML.)	HCT. (PER	BIL. (MG./100 ML.)	I.I.	C_{IN} (ML./MIN.)	P_{OSM} RANGE (MOSM/L.)	V RANGE (ML./MIN.)	$Tm^{c}_{H_2O}$ (ML./MIN.)	$Tm^{c}_{H_2O}/C_{IN}$ × 100	REGRESS-COEFF.	MAXIMAL OSMOTIC U/P RATIO¶	
														a	p
Sickle-cell anemia															
1*	12	M	1.20	6.0	16	1.4	–	141	278-278	1.83- 8.5	–	–	–	1.33	1.44
2*	7	F	0.73	8.1	–	4.1	–	200	283-310	0.69-16.0	11.5	5.72	1.120	1.43	1.59
3†	49	M	1.76	8.5	28	–	5	107	266-283	1.60-23.8	4.3	4.00	0.969	1.73	1.70
4*	35	M	1.69	9.8	29	–	–	114	282-295	2.36-27.5	3.4	3.00	0.916	1.59	1.58
5*	40	M	1.65	8.0	–	–	35	144	299-296	0.89-18.0	3.6	2.50	1.091	1.30	1.31
6*	47	F	1.60	7.0	–	–	17	76	290-298	2.00-13.9	1.91	2.54	1.039	1.36	1.38
											Mean	3.55	1.027	1.46	1.50
Anemia of other origin															
7‡	27	F	1.30	8.0	31	–	11	110	285-292	0.86-13.5	6.1	5.56	0.951	2.20	2.34
8§	29	F	1.29	8.5	27	–	–	143	275-279	0.20-14.3	6.6	4.60	0.831	2.10	3.10
9‖	68	M	1.77	5.9	22	–	10	93	316-305	0.78-12.0	4.37	4.72	1.011	2.69	2.54
											Mean	4.96	0.931	2.33	2.66

*Sickle-cell anemia.
†Sickle-cell trait; anemia due to bleeding hemorrhoids.
‡Macrocytic anemia of pregnancy.
§Microcytic anemia of pregnancy.
‖Chronic lymphatic leukemia.
¶a, before; p, immediately after Pitressin priming dose and before initiation of osmotic diuresis.

ing rate as $Tm^{c}_{H_2O}$. At urine flows less than $Tm^{c}_{H_2O}$, the osmotic U/P ratio is limited by a maximal value which in young, normal subjects reaches about 4.0.

The two functions, $Tm^{c}_{H_2O}$ and maximal osmotic U/P ratio, have been measured in 5 patients with sickle-cell anemia, in 1 patient with sickle-cell trait (A, S hemoglobin, no sickling, and anemia of short duration related to bleeding hemorrhoids), and in 3 patients with anemia not related to sickle-cell disease. The diagnosis was established either by hematologic examination alone or in combination with paper electrophoretic determination of the hemoglobin pattern. The highest plasma nonprotein nitrogen level observed was 32 mg. per 100 ml. (Patient 1). Mild albuminuria was present in only 3 patients (1, 2, and 6). Three patients (6, 7, and 9) showed occasional white blood cells in the urinary sediment.

MATERIALS AND METHODS

The patients studied were drawn from various Services of Bellevue Hospital. All were maintained on the regular hospital diet. Fluids were withheld for 15 to 18 hours before the test, which was carried out in the morning with the patient in the fasting state. Urine was collected by an indwelling catheter and residual urine was expelled from the bladder by air. No "washout" fluid was used. Control urine and plasma samples were collected for the determination of inuloid blank and oliguric osmotic U/P ratio after the patient was transferred from the ward to the research unit. A sustaining intravenous infusion of inulin in isotonic saline was then started at a rate of 1 ml. per minute after an adequate priming dose calculated to give a plasma concentration of about 25 mg. per 100 ml. A priming dose of Pitressin (1 milliunit per kilogram of body weight) was given intravenously and Pitressin was incorporated in the inulin infusion in such concentration that the rate of administration throughout the test was 1 milliunit per kilogram per hour.

After 2 or 3 urine collection periods for the determination of basal glomerular filtration rate, a progressively increasing osmotic diuresis was induced with 10 per cent mannitol solution infused at a rate of 10 to 20 ml. per minute. Urine was collected at 10-minute intervals according to the rate of urine flow and blood was collected 5 or 6 times during the test, which lasted for some 120 minutes after starting the infusion.

Inulin was determined by Schreiner's modification[12] of Roe's resorcinol method. The osmotic pressure of plasma and urine was measured with the thermistor, bridge, and null-point detector unit of Bowman, Trantham, and Caulfield[13], using a Johlin freezing-point apparatus.

RESULTS

Our data have been evaluated in the same manner as in previous reports from this laboratory[11, 14] and pertinent data are presented in Table I. Columns 1 to 8 of Table I need no comment. Column 9 shows the average inulin clearance before induction of osmotic diuresis, corrected to 1.73 square meter of body surface area. The inulin clearance increased (by 16 per cent) during osmotic diuresis in only one patient (Patient 3).

Columns 10 and 11 show the range in P_{OSM} and V as between the start of the test and the peak of osmotic diuresis. Column 12 shows $Tm^{c}_{H_2O}$, which is taken as the mean value of $T^{c}_{H_2O}$ calculated by equation I throughout the linear portion of the regression line of C_{OSM} on V, and neglecting deviations of the slope of this line from 1.0. A maximal value of $T^{c}_{H_2O}$ was not obtained in Patient 1 in whom the test was terminated early before reaching an adequate diuresis.

In column 13, $Tm^{c}_{H_2O}$ is factored by C_{IN} to permit comparison between different individuals and between the values reported here and those reported for normal subjects.[11]

Column 14 shows the slope of the regression line of C_{OSM} on V. The regression coefficient in our patients ranged above and below 1.0, but is constant throughout the test in each patient, as in normal subjects.[11]

Column 15 shows the osmotic U/P ratio observed in (a) the urine collected before and (p) in 3 samples collected from 15 to 60 minutes after the priming injection and start of the infusion of Pitressin.

DISCUSSION

The mean $Tm^{c}_{H_2O}/C_{IN}$ ratio in 5 patients with sickle-cell anemia and in one patient with sickle-cell trait is low (3.55), but within the range of normal values (5.0 ± 1.5).[11] However, the individual values observed in 3 adults with sickle-cell anemia (Patients 4, 5, and 6) are distinctly below the range of normal. The mean ratio of 4.96 observed in 3 patients with anemia of other origin is in good agreement with values observed in normal subjects. From these limited observations we infer that the maximal rate at which water may be abstracted in the concentrating operation, when measured during osmotic diuresis, is not specifically reduced by moderate anemia per se, early during sickle-cell disease and in sickle-cell trait, but is reduced late in sickle-cell disease. The low $Tm^{c}_{H_2O}/C_{IN}$ ratio suggests a specific defect rather than loss of functioning units, because it was demonstrated in chronic renal disease[14] that the ratio of $Tm^{c}_{H_2O}/C_{IN}$ does not change significantly with progression of the disease and loss of functioning tissue.

The presence of a specific tubular defect is furthermore suggested by the reduced osmotic U/P ratio, obtainable during oliguria, in all patients with sickle-cell disease, irrespective of age. The available data* on normal subjects indicate an upper limit in this value of 3.0 to 4.0,[16] whereas in our patients with sickle-cell disease this value is 1.73 or less (mean = 1.50). This ratio was not increased by Pitressin (column 15, Table I). In 3 patients with anemia of other origin but of similar severity, the maximal osmotic U/P ratio is higher (2.10 to 2.69) but still below the limits usually assigned to normal subjects.† These observations suggest that anemia per se can lower the osmotic U/P ratio obtainable during oliguria. However, the observations[10] that (1) urine osmotic pressure during oliguria is less than normal in patients with sickle-cell trait and no anemia and (2) that the defective concentrating mechanism can be reversed in infants (with sickle-cell anemia) by multiple transfusions of normal red blood cells, indicate that the marked lowering of the osmotic U/P ratio in sickle-cell disease is related to the presence of specific cells or hemoglobins rather than to the anemia.

In brief, this limited series indicates that in patients with sickle-cell disease, when tested during antidiuretic conditions, the maximal osmotic U/P

*It must be noted that these data are based on observations in relatively young persons. Miller and Shock[15] report that between the ages of 25 and 50 there is a reduction in the maximal inulin U/P ratio attainable during Pitressin antidiuresis but this datum does not exclude a possibly greater reduction in osmotic concentrating power.

†Patient 8 was the only one who showed an increased osmotic U/P ratio after Pitressin, but she was not very cooperative and may have taken fluids prior to the test.

ratio is reduced without substantial reduction of the maximal quantity of solute-free water which can be abstracted from the urine during osmotic diuresis. Our data do not clarify the nature of the defect which may be related to a diminished responsiveness to antidiuretic hormone or to a reduction of the attainable osmolar concentration gradient across the tubules.

SUMMARY

1. $T^c_{H_2O}$ and maximal osmolar U/P ratio were determined during hydropenia and the action of exogenous Pitressin in 6 patients with sickle-cell disease and 3 patients with anemia of different etiology.

2. In all patients with sickle-cell disease the maximal osmotic U/P ratio was low, while $Tm^c_{H_2O}/C_{IN}$ ratio was below normal in adults but within normal limits earlier in life.

3. It appears that a specific tubular defect is responsible for the low osmotic U/P ratio.

4. This defect in theory may be attributable to diminished responsiveness, of the tubules, to antidiuretic hormone, or to a reduction of the osmotic U/P ratio attainable during oliguria.

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LABORATORY METHODS

BIOLOGIC FALSE POSITIVE REACTIONS IN SEROLOGIC TESTS FOR SYPHILIS

Comparison of Standard Tests for Syphilis With the Treponemal Immobilization Test

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THE occurrence of biologic false positive (BFP) reactions in standard tests for syphilis (STS) has been well established and recently studied in certain diseases, employing the new techniques and antigens.[1-4] Obviously, latent syphilis cases are the type most difficult to differentiate clinically from individuals presenting such reactions. When dealing with the "chronic" BFP reactor,[5] this problem becomes nearly insoluble unless additional laboratory data are available. Fortunately, it is now possible to obtain such information by utilization of the treponemal immobilization (TPI) test.[6]

Use of the TPI test as an aid in the diagnosis of latent syphilis is well founded,[5, 7-12] but has raised questions relative to the proportion of BFP reactions occurring with any given test and in different population groups. Perusal of reports by other workers[9, 13-15] reveals that the standard Kahn test was employed in the selection of a large majority of the cases studied. For this reason, results on cases selected by means of other standard tests (VDRL and cardiolipin Kolmer) are presented. These are compared with the clinical findings and with the TPI test.

METHODS

Cases were accepted from anywhere in the U.S. Armed Forces in Europe and represent a cross section of such cases in this rather heterogeneous American group. Criteria for selection were: (1) absence of any history of syphilis, (2) no history of treatment for syphilis, (3) absence of any signs or symptoms of syphilis, (4) recent positive or doubtful STS reactions on 3 different specimens drawn at least a week apart, (5) no recent penicillin therapy for other disease, and (6) submission of a sterile serum specimen.

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Histories accompanied the serum from each case, stating the diagnostic problem. The presence of any known causes of "acute" BFP reactions was indicated. Cerebrospinal fluid examinations had been performed, with negative results, on 51 of the 183 cases studied, thus greatly reducing the likelihood of asymptomatic neurosyphilis in this portion of the series.

All cases submitted were included in the study if they met the criteria stated above. Sera were collected without preservative, were kept sterile, and were mailed or sent by courier to our laboratory where they were stored at approximately 5° C. until tested. Repeat specimens were tested from 25 cases. Sera from 169 cases were examined in this laboratory by the cardiolipin microflocculation test for syphilis. The cardiolipin Kolmer complement fixation test also was performed in 145 of the 169 cases. All sera from the 183 cases studied were examined by the TPI procedure, usually being tested 3 times. Except in the event of discrepancies, the STS were performed only once. All specimens were tested under code numbers and the histories were withheld from technicians performing the tests.

The TPI methods used were essentially the same as those described previously,[10] employing 18 hours' incubation at 35° C. and test mixtures in which 10 per cent of the final concentration was serum, 80 per cent was medium-treponeme suspension, and the remainder was guinea pig serum as a source of complement. When necessary, the amounts were adjusted slightly to permit addition of sufficient complement, but the final test serum dilution always was maintained at 1:10. Such minor adjustments are necessary in order to standardize the complement concentration at a level sufficiently high to ensure a speedy and sensitive reaction. This was done by titrating each guinea pig serum pool several times, using the spectrophotometric 50 per cent end-point method[16] and then calculating the approximate volume necessary to attain final concentrations of 90 to 120 units per milliliter in the reaction mixture. It was observed that this procedure aided greatly in maintaining reproducibility and in attaining sensitivity. A separate report dealing with these considerations is intended.

Controls used were the same as already described except that the positive control dilutions were twofold rather than threefold and 6 tubes were used, thus permitting graphical location of the IT_{50} titer[8] by 5 points rather than by 3. At least 2 known negative serum controls were included with each group of sera being tested.

The source of treponemes was rabbit testes inoculated with Nichol's strain *Treponema pallidum*. The rabbits were not kept in an air-conditioned room but the climate was quite mild. Rabbits developed typical orchitis in from 6 to 8 days. In some tests, frozen syphilomas were employed[17,18] and these usually yielded good results. The incidence of observable sensitization in vivo was almost nil.

TPI results were considered positive if 50 per cent or more of the treponemes were specifically immobilized. Tests yielding 20 to 49 per cent immobilization were considered doubtful, and those yielding less than 20 per cent immobilization were interpreted as negative.

The microflocculation (VDRL) test was performed as described in references 19 and 20, using the Boerner rotator, except that tests were reported as doubtful instead of "weakly positive" and cardiolipin antigen furnished by Walter Reed Institute of Research was used. This antigen is an ethanol solution containing 0.03 per cent cardiolipin, 0.27 per cent lecithin, and 0.90 per cent cholesterol. The complement fixation (CF) procedure followed the method of Kolmer,[21, 22] also using Army cardiolipin antigen. The CF antigen is an ethanol solution containing 0.0175 per cent cardiolipin, 0.0875 per cent lecithin, and 0.30 per cent cholesterol. Lack of complete hemolysis in the controls was interpreted as "anticomplementary." Such results are included in those listed as "not tested" in Table II. All procedures were performed in a routine fashion and no special consideration was given these specimens over other clinical sera tested concurrently.

RESULTS

Of the 183 untreated asymptomatic "seropositive" cases, 39 (22.5 per cent) yielded negative TPI tests. The distribution of results is shown in Table I. Only 8 of the 183 cases reported by other laboratories as reactive to the STS gave negative results on the specimen tested in our laboratory. One of these was positive by the TPI test. Since reported STS results usually were obtained from serum specimens drawn some days prior to the specimen tested in our laboratory and in view of the well-known fluctuation of STS results in latent syphilis cases, close comparison of such results with ours would not be of great value.

TABLE I. RESULTS OF SEROLOGIC TESTING OF 183 UNTREATED "SEROPOSITIVE" CASES

		OUR STS				TPI			
REPORTED STS		POSITIVE	DOUBTFUL	NEGATIVE	NOT DONE	POSITIVE	DOUBTFUL	NEGATIVE	UNSATISFACTORY
Positive*	131	103	16	3	9	109	1	20	1†
Doubtful	52	14	28	5	5	31	2	19	0
Total	183	117	44	8	14	140	3	39	1

*The STS was considered positive if either VDRL or CF was positive; negative only if both were negative; all others were considered doubtful.
†Serum immobilized treponemes in the absence of complement.

Comparisons of our results using the 3 procedures employed in this study are presented in Table II. Of the 169 cases retested by the VDRL procedure in our laboratory, 161 yielded positive or doubtful microflocculation tests, while 31 (19 per cent) were negative by the TPI procedure. Results with the complement fixation test were obtained on 145, of which 92 (63 per cent) were positive or doubtful and 53 (37 per cent) were negative. The former yielded 9 (10 per cent) negative TPI tests, while the latter yielded 25 (47 per cent) positive TPI tests. The implications of this will be discussed later.

TABLE II. COMPARISONS OF OUR STS RESULTS (USING CARDIOLIPIN) WITH THE TPI RESULTS

				TPI		
VDRL		KOLMER CF		REACTIVE	NEGATIVE	UNSATISFACTORY
Reactive	161	Reactive	91	83	8	0
		Negative	46	24	22	0
		Not tested	24	22	1	1
			161	129	31	1
Negative	8	Reactive	1	0	1	0
		Negative	7	1	6	0
			8	1	7	0
Not tested	14	Not tested*	14	13	1	0
Total	183		183	143	39	1

*Includes anticomplementary results.

There were far fewer doubtful results with the TPI test than with either of the other tests used. Repeat tests on the same serum, performed on an average of 3 times for each specimen, were in almost perfect agreement. There were only 12 tests showing appreciable variation out of a total of approximately 600 tests performed and these results were clarified on repeat testing.

In addition, results with 25 repeat specimens (different sera) agreed in every case with the original specimen. There were about 11 per cent of the tests which were considered unsatisfactory because of factors such as poor survival in controls, contamination, etc., but these results were clarified upon repeated testing except in one instance.

Examination of the case histories revealed that only 15 of the 183 patients had clinically recognizable disease or were pregnant. Of the 15, all but 3 yielded positive TPI tests. The 3 TPI negative cases were myxedema, convalescent infectious hepatitis, and chronic cholecystitis. All patients in this study had received routine Army immunizations some time previously but in only one case was this cited as a possible cause of a BFP reaction. This patient's serum was positive by the TPI test.

DISCUSSION

In attempting to determine the proportion of STS reactions which may be false, the main difficulty has been the lack of a standard of reference which would permit the differentiation of true latent syphilis. It has now been established that the TPI test is a dependable means of attaining this. Both the specificity and the sensitivity have been shown to be quite high.[7, 8, 23] Providing that no early or treated cases of syphilis are included, the TPI procedure yields very few doubtful reactions and therefore provides a clear-cut differentiation. In the presence of positive or doubtful STS, where all clinical evidence and history referable to syphilis are absent,[10, 11, 13] the TPI negative cases may be assumed to be BFP reactors to the STS and the positives to be cases of latent syphilis. Of course, it is not implied that such a procedure, even though statistically valid, should be applied in quite the same manner to individual case diagnosis. For such purposes, laboratory tests may be depended upon as diagnostic aids only in the presence of prescribed clinical circumstances.

In view of the foregoing, it appears that the majority of those "seropositive" cases yielding negative TPI test results were false positive reactors to the STS when cardiolipin antigen was employed. It is unlikely that an appreciable proportion of latent syphilis cases were overlooked because all diagnosed cases of late or latent syphilis tested during this period in our laboratory have yielded positive results and, furthermore, positive control sera supplied by the serologic standardization section of the World Health Organization have yielded reproducible results indicating high test sensitivity.

The fact that only 3 of the TPI negative patients had clinically recognizable disease and that all were reactive on several occasions to the STS, leads us to conclude that the large majority of the false positive cases observed were "chronic" BFP reactors.

It can be seen from Table II that sera reactive to the microflocculation test yielded a higher proportion of false results (19 per cent) than did those which were reactive to the complement fixation procedure (10 per cent). A glance at the TPI results of sera negative to the CF test (Table II) shows that

much of the apparent difference may be due to low sensitivity of the complement fixation test against those sera. Thus the CF cannot be recommended for sole use as an exclusion procedure, lest this result in failure to detect about 15 per cent of latent syphilis.

The careful selection of cases and the requirement for repeated STS screening, in addition to the other considerations discussed above, could be expected to eliminate practically all but "chronic" BFP reactors and latent syphilitics. It might be argued that this may have contributed to the lower incidence of BFP reactions reported herein, as compared with those reported by Moore,[13] 45.3 per cent; Harrell,[9] 58.4 per cent; Nelson,[15] 42.5 per cent; and Miller,[14] 55.6 per cent. It is more likely, however, that the main reason for the difference between our findings and the findings of others is the use of the cardiolipin microflocculation test as a screening procedure in the selection of these cases. It has been shown previously that the standard Kahn test is more likely to give false or oversensitive reactions than is the VDRL procedure.[1, 3]

SUMMARY

Cases presenting a problem of differentiation between latent syphilis and a chronic BFP reaction were collected, retested by the STS, and examined by the TPI test. Of 183 such cases studied, 39 (22.5 per cent) were negative by the TPI test. It is concluded that the incidence of BFP reactors, when screening of asymptomatic "seropositive" cases is performed with cardiolipin, is less than when such screening is done with other antigens.

The Kolmer complement fixation test using cardiolipin antigen yielded fewer false positive reactions but lacked sufficient sensitivity to be used for exclusion purposes, due to the large proportion of latent syphilis which would be missed in such a procedure.

ADDENDUM

While these data were being prepared for publication, another report of similar findings was made (MacPherson, D. J., Ledbetter, R. K., and Martens, V. E.: Am. J. Clin. Path **25**: 89-92, 1955. These authors observed 29.4 per cent false positive reactions in tests performed with cardiolipin antigen. Examination of their results reveals that 21 per cent of these reactions were of the "acute" type rather than "chronic" reactors, such as those examined in our study. Adjustment of their figure to correct for this yields 23 per cent "chronic" BFP reactors, an observation almost identical with ours.

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EVALUATION OF THE GRIESS NITRITE TEST AS A METHOD FOR THE RECOGNITION OF URINARY TRACT INFECTION

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INTRODUCTION

A ROUTINE screening test for the rapid, dependable diagnosis of urinary tract infection would be a welcome adjunct to the diagnostic armamentarium of the physician. In a recent report by Schaus,[1] the Griess nitrite reaction was suggested as fulfilling this need. The reaction depends on the presence of infecting bacteria which produce nitrite from nitrate usually found in the urine. Most of the organisms causing urinary tract infections, including *Escherichia coli*, Proteus, Micrococcus, and Aerobacter will perform this reduction; the tubercle bacillus, streptococci, and gonococci will not.

The purpose of this paper is to report observations made on the quantitative aspects of the reaction which indicate its limited usefulness as a screening or diagnostic procedure.

MATERIALS AND METHODS

A. *Bacterial Strains.*—The microorganisms employed were strains of *E. coli*, *A. aerogenes*, Pseudomonas species, and paracolon recently isolated from the urine of patients with pyelonephritis. These were chosen since they represent the most common nitrite-producing bacteria found in urinary tract infections.

B. *Reagents.*—The Griess reagent was prepared according to Schaus. Sulfanilic acid (C.P.), 0.5 Gm., was dissolved in 150 ml. of 10 per cent acetic acid and 0.2 Gm. of alpha-naphthylamine (C.P.) was dissolved in 20 ml. of boiling distilled water, thoroughly stirred and filtered. These 2 solutions were mixed and then stored in a dark bottle. The freshly prepared reagent is a colorless solution; but over a period of a few weeks a pink color develops which interferes with the test and necessitates preparation of a fresh solution.

C. *Test for Presence of Nitrite.*—In the presence of nitrite, a red color rapidly develops upon the addition of the Griess reagent due to the formation of azo-α-amidonaphthalene-parazobenzene-sulfonic acid.[2] This is a relatively stable compound, the color persisting for several hours.

The test may be carried out in one of two ways: (1) addition of several milliliters of Griess reagent to 2 to 4 times this amount of freshly voided urine (or broth containing bacteria), (2) addition of one drop of Griess reagent to 3 drops of urine (or broth) on a white spot test plate. In the experiments subsequently described, when serial tests were made of urine or broth cultures for the presence of nitrite, the spot test plate technique was employed. During preliminary work, both methods were simultaneously tried on the same specimens and no difference was noted, i.e., both methods became positive at the same time and developed the same intensity of color.

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Serial tenfold dilutions were made in saline with 1 ml. of a 4-hour broth culture of the bacterial strain to be tested, and enumeration of the colony count was obtained from these dilutions by standard techniques. In evaluating the rate of nitrite production by different numbers of organisms of a particular bacterial strain, a 1 ml. aliquot of the desired tenfold dilution was transferred to a tube containing 8.7 ml. of beef heart infusion broth and 0.3 ml. of a 10 per cent sodium nitrate solution. This was designated "time zero" and the tubes incubated at 37° C. At 30-minute intervals, spot plate tests were performed to determine the presence of nitrite. When the test became positive, bacterial enumeration was again performed. Thus, with this procedure, the initial concentration of organisms, the time required for the production of a positive nitrite test, and the bacterial population estimate at which the test became positive could be determined.

RESULTS

A. *Sensitivity of the Test.*—In order to determine the sensitivity of the Griess reaction, serial dilutions of known concentrations of sodium nitrite were tested with the spot plate and larger volume techniques. As shown in Table I, the test was positive at 0.1 μg concentrations of sodium nitrite and negative at 0.01 μg.

TABLE I. SENSITIVITY OF THE NITRITE TEST

TUBE NO.	SODIUM NITRITE CONCENTRATION MG./ML.	GRIESS TEST
1	10	Positive
2	1.0	Positive
3	0.1	Positive
4	0.01	Positive
5	0.001	Positive
6	0.0001	Positive
7	0.00001	Negative

B. *Determination of Time Required by a Known Concentration of Nitrate-Reducing Organisms to Produce a Positive Nitrite Reaction.*—To evaluate the rate of nitrate reduction by a particular strain, different dilutions of broth cultures were inoculated in broth-nitrate mixture, incubated at 37° C., and tested at intervals with the Griess reagent. As seen in Table II, an initial concentration of 1,050,000 viable units of *E. coli,* and 4,800,000 of *A. aerogenes* was required to produce detectable nitrite in 2 hours. Smaller inocula required even longer periods. A second strain of *E. coli* with 1,020,000 viable organisms required 3 hours to produce detectable nitrite. There appears to be some variation in the rate of nitrate reduction by the different organisms, not related to the rate of growth. Thus, 48,000 *A. aerogenes* produced enough nitrite to give a positive test in 5 hours at which time there were 65,200,000 viable units per milliliter of culture, while 40,000 Pseudomonas units produced a positive Griess reaction in 7½ hours with a final concentration of 17,000,000, and 40,000 paracolon organisms yielded sufficient nitrite to be detected in 5 hours at a time when the number was 10,200,000. Indeed, the 2 strains of *E. coli* tested demonstrated this variability. Although both initial concentrations were a little more than 1,000,000 organisms per milliliter, one became positive in 2 hours at a final concentration of 34 million and the other in 3 hours with 82 million viable units present.

To determine whether the broth employed in these experiments interfered with the nitrite test, urine was obtained from a normal subject, sterilized by passage through Seitz porcelain filters (No. 2), and used in similar experiments. Table II shows the results of such incubation in urine with a strain of *E. coli* simultaneously tested in broth. It is noted that the Griess test became positive at a slightly later time in urine, and that multiplication of the organisms was less rapid in urine than in broth.

TABLE II. TIME REQUIRED FOR VARYING NUMBERS OF BACTERIA TO PRODUCE DETECTABLE NITRITE

ORGANISM	INITIAL CONCENTRATION	TIME REQUIRED FOR POSITIVE GRIESS TEST	FINAL CONCENTRATION
A. INCUBATION IN BROTH			
E. coli (strain 1)	105	7½ hours	–
	10,500	4½ hours	35,000,000
	1,050,000	2 hours	34,000,000
E. coli (strain 2)	102,000	4 hours	83,000,000
	1,020,000	3 hours	82,000,000
	10,200,000	1 hour	23,800,000
A. aerogenes	480	7½ hours*	–
	48,000	5 hours	65,200,000
	4,800,000	2 hours	59,000,000
Pseudomonas	48,000	7½ hours	17,000,000
Paracolon	400	8 hours	41,000,000
	40,000	5 hours	10,200,000
B. INCUBATION IN URINE			
E. coli (strain 2)	102,000	5 hours	14,600,000
	1,020,000	3½ hours	12,500,000
	10,200,000	2 hours	20,800,000

*The Griess test was still negative at 7½ hours at which time testing was terminated.

DISCUSSION

A reliable screening test must fulfill 2 desirable properties: (1) it must exhibit a small number, if any, false positive reactions and (2) even more important, particularly when dealing with urinary tract infections, the number of false negative reactions should be extremely small, ideally zero. The question of false positive reactions is not a major consideration since Jaeggy and Lanz[3] stated that they had never found nitrite to be present in urine which was sterile. Provided the test is carried out on a freshly voided urine sample, then, the incidence of false positive reactions should be zero.

Schaus proposes 4 causes for the occurrence of a negative reaction aside from a sterile urine or infection with a nonnitrate-reducing micro-organism. These are (1) deterioration of the reagent, (2) no nitrate present in the urine to act as a substrate, (3) voiding so frequently that bacteria lack necessary time to reduce nitrate, and (4) urinary retention causing the nitrite to be further decomposed by the same bacteria. He states that causes 2, 3, and 4 can be eliminated by adding several drops of 10 per cent sodium nitrate solution to the urine and incubating for 2 hours at 37° C. This, he suggests, will produce a positive Griess test provided nitrate-reducing bacteria are present. Our

results show, however, that although the test will become positive when a high concentration of organisms is present (1,000,000 per milliliter or more), lower concentrations require a much longer time to produce detectable nitrite. These figures are particularly significant since bacilluria of 10,000 or more organisms per milliliter is probably indicative of urinary tract infection.[4, 5] Urine containing bacteria in such quantity would exhibit a negative Griess test, even with incubation in the presence of excess nitrate. In a large clinical study of 260 urines infected with nitrate-reducing bacteria, Bechgaard and Jansen[6] found the nitrite test positive in only 69 per cent of cases. An incidence of 31 per cent false negative reactions would also indicate that the test is not a reliable one for use as a routine screening procedure. The real need for a test of this sort is in the recognition of the more subtle urinary tract infections and it is in this realm that the Griess nitrite reaction leaves much to be desired.

SUMMARY

The reliability of the Griess nitrite test in the recognition of urinary tract infection has been evaluated, employing several micro-organisms known to reduce nitrate. Although the Griess reaction is able to detect 0.1 μg of sodium nitrite per milliliter, it appears to be of limited value as a screening or diagnostic test for the presence of urinary infection. The inability of some pathogenic bacteria to reduce nitrate, the variable rate of the reaction with other organisms, and the relatively large numbers of microorganisms required to produce detectable nitrite detract from the value of this test as a practical clinical procedure.

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STANDARD CLOTTING TIME

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THE determination of the clotting time on whole blood was one of the first tests used in hematology. However, the large number of different techniques has created considerable confusion and uncertainty in the proper evaluation of this test in normal and pathologic conditions. Even the well-known and widely used Lee-White method has been shown to give varying results due to numerous modifications of the original technique and to subjective differences in the performance of the test.[1]

After careful study of all possible sources of error, we have adopted the following standardized procedure to obtain highly reproducible clotting time readings on unmodified whole blood.

EQUIPMENT

1. Ten milliliter all glass Luer-Luk syringe.

2. Sharp 19 gauge stainless steel intravenous needle with short bevel.

3. Three-way stopcock (Becton, Dickinson No. 5031/S or L/S2 with left- or right-handed side arm according to the preference of the operator). A special adapter* holding 3 capillary tubes is inserted in the "female" Luer slip of the side arm. This adapter (Fig. 1) will permit the simultaneous filling of 3 tubes.

(Items 1 to 3 are washed with Haemo-Sol, tap water, distilled water, and autoclaved for each determination. The valves of the stopcocks are cleaned and oiled weekly with Dow Corning Silicone oil No. 200.)

4. Glass capillary tubes of 100 mm. length and 1.6 to 1.8 mm. diameter.† One end of each tube is marked with a small piece of adhesive tape before usage.

5. Plasticine.

6. Stop watch.

7. Water bath constructed from a large thermos flask (4¾ inches inside diameter) with a rubber cover. As this type of flask is not available with an insulating protective case, it is surrounded by a Fiberglas insulating material and placed in an aluminum container of adequate size with a carrying handle. This water bath can easily be transported to the bedside. The flask is filled with 2 L. of water which is maintained at 21 ± 0.1° C. by the addition of either hot or cold water. To keep the capillary tubes from sinking to the bottom, we have constructed a simple rack out of hard rubber tubes and copper wire to fit in the flask. A plastic grid covered with 2 layers of gauze is placed on top of the rack. The flat top of the rack is about 3 cm. under the water level.

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*Available from R. D. Bradfield, 421 Earl Street, Kingston, Canada, and from Becton, Dickinson and Company, Rutherford, N. J.

†Melting point capillary tubes No. 12-141 from Fisher Scientific Co.

8. Mercury thermometer possessing one-tenth degree subdivisions to check the temperature of the water bath.

9. A small (1 × 2 × 3 cm.) Y-shaped rubber block (Fig. 2).

10. In hospitals where clotting time tests are performed frequently, a small food-tray wagon is very convenient for carrying all the necessary utencils. This allows the operator to stop anywhere to manipulate the capillary tubes.

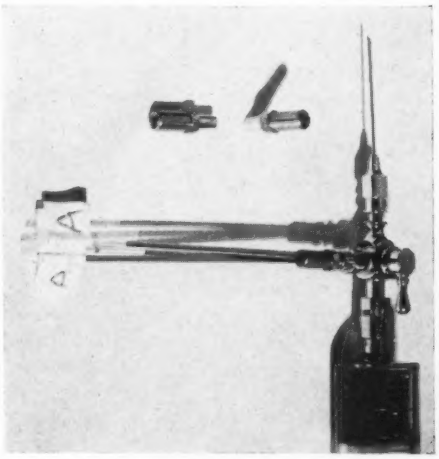

Fig. 1.—Assembled syringe, stopcock, needle, adapter, and capillary tubes filled with blood after removal from the vein. Two adapters are seen separately in the upper part of the figure.

Fig. 2.—Breaking of a capillary tube held in a Y-shaped rubber block.

METHOD

1. Assemble syringe, stopcock, needle, adapter, and 3 capillary tubes as seen in Fig. 1.

2. After application of a tourniquet on the arm, a neat venipuncture is made and approximately 5 to 8 ml. of blood drawn slowly into the syringe. This blood is *not* used for the test.

3. Deflect the flow from the needle into the side arm by turning the valve slowly, thereby filling the 3 capillary tubes simultaneously to within 1 cm. of the end. It is important not to close and reopen the valve while blood is flowing into the tubes. Finally the valve is closed and the whole set is withdrawn from the vein. Special attention

should be paid to keep the needle immobilized in the vein while operating the stopcock and collecting the blood. If the needle slips out, one should start the whole test again with autoclaved utensils and a new venipuncture on the other arm.

4. The stop watch is started when the blood enters the capillary tubes.

5. Both ends of the capillary tubes are sealed immediately with plasticine and placed horizontally in the water bath.

6. About 3 or 4 minutes before the expected end point, one capillary tube is taken from the water bath with a forceps, and a small piece is broken from the end which the blood entered first (opposite the tag). Each breaking procedure should not last more than 10 to 15 seconds. An important temperature difference is produced by the fingers of the operator. To avoid this, the capillaries are grasped by the adhesive tag and placed in the Y-shaped rubber block (Fig. 2). Holding the rubber block, small pieces can easily be broken off with the thumb and index finger of the other hand without touching the remaining part of the tube with the bare fingers (Fig. 2).

7. The end point of blood clotting is marked by the presence of a thin yellow fibrin thread which is easily seen against a white background. The first fibrin thread is very fragile and breaks easily. In 20 to 30 seconds its resistance increases. When a 2 cm. thread can be drawn, the arbitrary end point has been reached. If the end point is exceeded the fibrin thread is covered with little beads of blood and later the clotted whole blood can be pulled out of the tube. The blood droplets on the filament signify that the arbitrary end point is just passed, while the thick blood clot signifies considerable overtime.

The determination of the end point is usually very easy. Occasionally one may find that the very first break of the tubes does not produce a fibrin thread and that a second break made immediately after the first shows a fibrin thread of 2 cm. length. This can be explained by the fact that at the beginning the fibrin is not anchored to the glass wall of the tube and hence pulling apart the broken pieces will not demonstrate an appreciable fibrin thread. However, the sharp edge of the broken end catches the fibrin and a subsequent break produces a fibrin thread of adequate length. If the end point is not attained the tube is resealed and replaced as quickly as possible in the water bath. The procedure is repeated every 30 seconds with alternate capillaries until the end point is obtained in all the 3 tubes.

8. The average of the readings of the 3 capillaries is the standard clotting time.

9. If a marked elevation of clotting time is suspected, 3 capillary tubes will not be enough to complete the test. In this case it is necessary to assemble 2 or 3 stopcocks in order to permit the filling of 6 or 9 tubes. During the filling procedure it is important to open the stopcocks in the following sequence: No. 1, thereafter No. 2, and finally No. 3 (Fig. 3).

RESULTS

1. Most of the steps of the method are self-explanatory. However, we found it worth while to demonstrate the importance of certain aspects of the technique.

a. *The amount of blood aspirated before filling the capillary tubes:* In 7 patients under anticoagulant treatment the standard clotting time was determined after removing 1 to 10 ml. of blood. Three to five serially connected stopcocks were used as seen in Fig. 3. The curves in Fig. 4 show that increasing the amount of blood removed also increased the clotting time; however, the removal of more than 5 ml. of blood did not alter the clotting time. Patients 1 to 5 had obese arms. Curves No. 6 and 7 were obtained from patients with thin subcutaneous tissue and bulging veins. In these 2 cases the clotting time was not increased after removal of more than 2 ml. of blood.

b. *Difference between readings obtained from capillary tubes broken with bare fingers and those held in rubber block:* Blood was taken from 10 healthy adults filling 6 tubes on each occasion. Three tubes (Group A) were broken with bare fingers, the others (Group B) by holding the tubes in a rubber block.

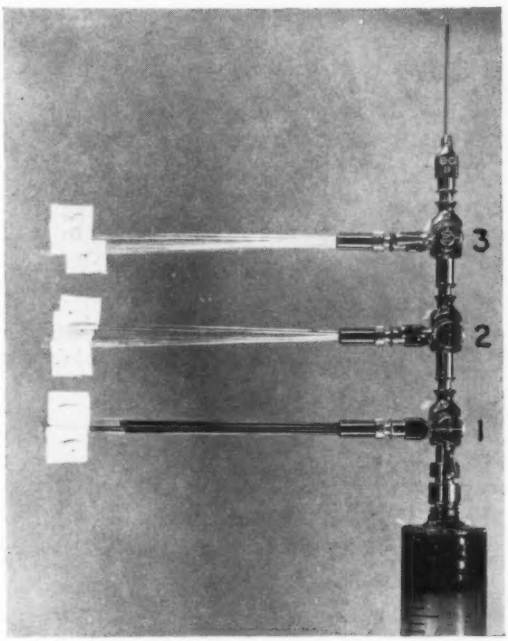

Fig. 3.—Serially assembled stopcocks for filling 9 capillary tubes. Valve No. 1 is opened and the first 3 tubes filled with blood; thereafter valve No. 2, finally No. 3 is opened.

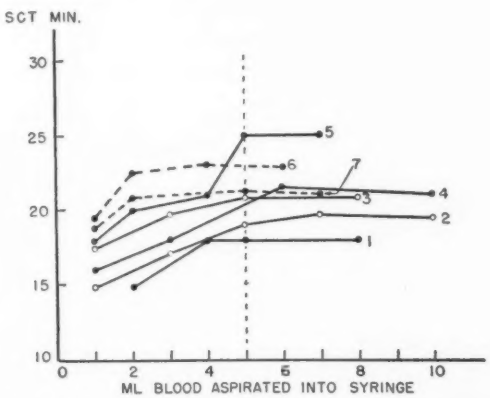

Fig. 4.—Standard clotting time readings after removal of increasing amount of blood before filling the capillary tubes. Aspiration of more than 5 ml. of blood did not alter the clotting time. Curves No. 6 and 7 from patients with thin subcutaneous tissue show no increase of standard clotting time after removal of more than 2 ml. of blood.

The mean of the clotting times of Group A was 9.6 minutes (s.e. ± 0.45 minute) and that of Group B 11.8 minutes (s.e. ± 0.13 minute) the difference being highly significant.

2. *Normal Values.*—In 40 healthy men (23 to 35 years of age) the test was performed after overnight fasting. The mean of the readings obtained in 3 capillary tubes was 11.99, 11.94, and 11.88 minutes with standard deviations of ±0.92, ±0.96, and ±0.87. The difference between the individual tubes was not significant.

3. *Reproducibility of the Method.*—In the same healthy young man, we performed the test on 12 occasions after an overnight fast. The mean of the readings was 11.48 minutes, standard deviation ±0.45, and standard error ±0.13. The last figure can be considered as the standard error of the technique.

4. *Clinical Observations.*—It was found in 289 patients treated by various oral anticoagulants that the standard clotting time was significantly elevated if the dosage of drugs was adequate.[10]

Abnormally high standard clotting time readings have been demonstrated in 4 patients with hemorrhagic disorders. More elaborate tests revealed plasma thromboplastin component (PTC) deficiency in 2 cases, von Willebrand-Jürgens syndrome in one case, and an excessive amount of circulating antithrombin in another case. In 3 of these cases our test served as a useful guide during therapy.

DISCUSSION

The clotting time of unmodified blood in vitro is influenced by (1) the constituents of the blood, (2) admixture of tissue juice, (3) temperature at which the test is performed, and (4) the foreign surface with which the blood is in contact during the procedure. The ideal method for determination of of the clotting time should measure the interaction of blood constituents on the coagulation only. In order to achieve this goal, the above-mentioned external factors should be eliminated or meticulously standardized.

In accordance with other workers, we have found that the major source of error in the different clotting time methods is the mixture of tissue juice with the blood. Even venous blood collected by simple venipuncture contains a variable amount of tissue juice. This source of error is avoided in our procedure as the first 5 to 8 ml. of blood is not used and the blood which passes into the capillary tubes is relatively free from disturbing tissue juice. In spite of the fact that in patients with thin subcutaneous tissue and superficial veins 2 to 3 ml. of blood is sufficient to flush the needle, in routine work the aspiration of 5 to 8 ml. of blood is necessary. This amount is enough in every case to remove the tissue juice from the needle.

Since the coagulation of blood involves stoichiometric and enzymatic reactions, it is obvious that the environmental temperature must significantly influence the speed of the process. Several authors[3-6] have demonstrated clearly the inverse relationship between clotting time and environmental temperature. Most recommend body temperature (37° C.), claiming that this is the temperature at which coagulation takes place physiologically. We had the following objections to this proposal. First, for maintaining a 37° C. temperature, one would need an electric water bath transported to the bedside, while a lower temperature such as 21° C. can be easily maintained in an

inexpensive large thermos flask. Second, at 37° C., the clotting time is too short to detect minor differences. Fowler[7] has noted that 10 minutes after intravenous injection of 25 mg. heparin, the Lee-White clotting time at room temperature was prolonged, but it was within normal limits when the test was carried out at 37° C. Therefore, we adopted the temperature of 21 ± 0.1° C. for the water bath. Early in our research[8] we did not realize the importance of using insulating material between the fingers and capillary tubes during the breaking procedure. The comparative tests have shown that when the tubes are held in a rubber block a significant shift (2.5 minutes) of readings occurred toward a higher range. At the same time, the standard error of the technique decreased from ±0.45 to ±0.13 minute, demonstrating a greater accuracy of this technique.

The effect of foreign surfaces (syringe, test tube) on clotting time has been clearly demonstrated.[1-3] In our method the extent of blood-glass contact is standardized by the use of capillary tubes of similar size and by exclusion of the variable effects of syringe and test tubes.

The mean clotting time of 40 healthy men with our technique was 11.9 minutes with a standard deviation of ±0.9 minute. The range of normal values is somewhat greater than we reported earlier.[8]

Repeated tests in the same healthy subjects showed that the standard clotting time is fairly constant for the individual and can be determined with high reproducibility. The standard error of the technique was ±0.13 minute. In other words, the 95 per cent fiducial limits of each determination using 3 capillary tubes are ±0.38 minute (about ±25 seconds). This accuracy is considerably higher than that of many commonly used hematological tests.

We have been able to demonstrate that oral anticoagulants in adequate dosage do prolong the clotting time of whole blood, and that the Quick one-stage prothrombin test has poor correlation with the standard clotting time.[9] This test was found in 289 patients to be superior to the Quick one-stage prothrombin test in the clinical control of various oral anticoagulants.[10]

Using the standard clotting time as a screening procedure we have detected 4 cases of various disorders of the blood clotting mechanism. We strongly feel that our test will facilitate quick screening of potential hemorrhagic disorders and permit easy follow-up of their treatment.

Hitherto the clotting time has been considered to be unreliable and frequently a misleading laboratory test. Many attempts to standardize methods have resulted in an almost complete failure. However, by the meticulous elimination of possible sources of error, we have demonstrated that the standardized method presented by us is a highly reproducible and reliable test in the study of normal and pathologic blood clotting.

SUMMARY

1. A standardized test for determination of the clotting time is described. After a short period of training, it is easily performed with inexpensive material and gives reproducible results in absolute terms.

2. The standard error of the technique is ±0.13 minute.

3. The normal value in healthy young men is 11.9 minutes, standard deviation being ±0.9 minute.

4. Since subjective error is minimal, control blood for reference values is superfluous and the comparison of the results of different workers is possible.

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A NEW METHOD FOR RAPID PRECISE DETERMINATION OF CARBON MONOXIDE IN BLOOD

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CARBON monoxide, with an affinity for hemoglobin about 250 times greater than that of oxygen, is the most important gaseous poison and, in civilian practice, accounts for more deaths than all the other gases combined.[1] The relative stability of carboxyhemoglobin also makes this gas a fascinating tool for physiologic research and for certain laboratory tests including, for example, indirect measurement of arterial oxygen pressure, determination of total heme pigments and "true blood volume," and measurement of the diffusion capacity of the lungs. Some of these techniques fell into disrepute and were replaced by less pertinent or more complex tests chiefly because of the great difficulties of precise analysis of carbon monoxide in blood.

The usefulness of qualitative methods for estimation of carboxyhemoglobin[2] is limited to detection of severe carbon monoxide poisoning while spectroscopic methods are most accurate with relatively high concentrations of the gas, require special and complex apparatus and, under certain circumstances, may not be entirely specific.

The two most common methods in present use are the manometric method of Van Slyke and associates[3] and the microvolumetric syringe method of Scholander and Roughton.[4] Although an accuracy of 0.05 volume per cent COHb can be obtained in experienced hands, both methods are technically difficult, time consuming, and of decreasing accuracy with low carboxyhemoglobin concentrations.

Precise determination of CO in gas mixtures formerly presented even greater problems than the determination of CO in blood. Recently, highly sensitive physical techniques for CO gas analysis have made possible the estimation of CO concentrations with an accuracy of one part per million. Adoption of these methods for blood CO analyses was a logical development.

With conventional industrial indicators, CO is oxidized to CO_2 with the catalyst, Hopcalite, and the liberated heat is measured during regulated flow

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of the test gas. Such a meter, modified to read to an accuracy of 0.0005 per cent CO,[5] was used by Siösteen and Sjöstrand[6] for blood CO analysis. Unfortunately, for high accuracy, at least 5 L. of gas and 20 ml. of blood were needed and the action of the meter was not entirely specific because heat is developed by other oxidizable gases. We have found it impossible to remove all CO from the solution by brief exposure to oxygen without vacuum extraction. The volume of wash-gas has since been increased to 10 L.[7] and extraction time prolonged to as much as one and one-half hours.[8]

Another type of physical CO analyzer is the recently perfected, nondispersion, infrared meter of the positive type based on the principle of Luft.[9] It is simple, highly specific, more sensitive than spectrographic instruments and, with certain precautions, can be made to read to 0.00005 per cent CO with a sample of only 150 ml. An analyzer of this type was used for COHb determination by Lawther and Apthorp.[10] Except for the need of a special instrument and pump for blood gas extraction and the requirement for a relatively large blood sample, this system appeared attractive.

At our laboratory, we required a simple and accurate method for carboxyhemoglobin determination for evaluation of CO "back pressure" during pulmonary diffusing capacity measurements. The technique to be described permits determinations of blood CO content with a high degree of accuracy, which does not diminish with decreasing COHb saturations, and requires only a small quantity of blood. In addition to an infrared gas analyzer, only the usual Van Slyke manometric apparatus is needed and the technique can be mastered in a few minutes.

METHOD

Principle of the Method.—A measured volume of blood is introduced to the chamber of a manometric Van Slyke apparatus, the hemoglobin is converted to methemoglobin, and extraction is made in the usual manner. The extracted gas is diluted with room air and transferred to a tonometer. The CO concentration of the gas is measured with an infrared analyzer. The CO content of the blood, in volumes per cent, can be calculated from the known volume of blood sample, volume of tonometer, and CO concentration in the tonometer gas.

Treatment of the Blood Sample.—The choice of arterial or venous blood depends upon the design of the study. For detection of CO poisoning or blood volume determination, a venous sample suffices. Arterial blood is required during CO breathing when the carboxyhemoglobin saturation must be known at any given moment. Blood is drawn into a heparinized syringe, mercury is added, and the tip is plugged in the usual way.

Extraction of Gas.—The equipment required includes a Van Slyke–Neill stopcock pipette, a Van Slyke manometric apparatus, and a mercury-filled tonometer, fitted with glass stopcocks at the top and bottom, connected to a leveling bulb. The volume of the tonometer must be determined accurately initially.

Experimentally, two different solutions were used for decomposition of the blood: (1) the acid-ferricyanide solution, recommended by Siösteen and Sjöstrand,[6] prepared with 92 volumes of 32 per cent $K_3Fe(CN)_6$ and 8 volumes of concentrated lactic acid, and (2) 5 per cent sulfuric acid, as recommended by Dahlström.[8] The decomposing solution need not be deaerated and can be stored indefinitely.

Well-mixed blood is transferred anerobically in the usual manner to a rubber-tipped Van Slyke–Neill pipette.[11] The Van Slyke apparatus is prepared with caprylic alcohol,

4 ml. of decomposing solution are placed in the cup, and one-half milliliter is admitted to the chamber. The contents of the pipette are transferred to the chamber followed rapidly by 3 ml. of the solution from the cup. The stopcock is sealed and the solution is evacuated and shaken as for any other blood gas extraction.[11] No manometric readings are made, and c-corrections and blank determinations are unnecessary. Accurate volumetric transfer of the blood is essential since this appears to be the limiting factor in the precision of the technique.

After evacuation, the chamber is shaken 4 minutes. The side arm of the main-entry stopcock is then connected to the mercury-filled tonometer with a short length of plastic tubing. The vacuum is released with the leveling bulb in the lower position and room air is admitted through the cup until the chamber is filled with gas; the gas is transferred to the tonometer by raising the leveling bulb to the upper position. With the first extraction secured the chamber is sealed, vacuum reapplied, and shaking continued for three minutes; the dilution and transfer process is then repeated. More air is admitted into the chamber and transferred directly to the tonometer to flush chamber and tubing. The upper cock of the tonometer is closed, the connecting tubing removed, and its leveling bulb lowered until the pressure in the tonometer is slightly lower than atmospheric. With this reduced pressure maintained, air is slowly admitted through a slightly opened cock until the mercury level reaches the lower cock. The top cock is then quickly closed, the leveling bulb elevated, and the sample is stored under positive pressure. It is important that the gas be at atmospheric pressure at the moment when the mercury reaches the lower cock and the upper cock is closed.

The Infrared Meter.—A Liston-Becker Model 15 gas analyzer* was modified by addition of an external 7-inch mirror-scale milliammeter, which can be read to 0.2 per cent of full scale.† This is shunted so that a full-scale deflection (1 Ma.) is obtained from a signal which causes the built-in meter to read half scale (when the external meter connection is short-circuited). The larger scale and the shunting in 2:1 ratio quadruples the useful accuracy of the instrument.

The inlet of the sampling cell is fitted with a 25 ml. drying tube containing 8-mesh indicator anhydrous calcium sulfate. Unless an adequate cotton filter is used to prevent entrance of particles into the cell, a marked zero shift and reduction in sensitivity occurs with every rapid flow of gas.

Calibration and Preparation of the Meter.—The response of the instrument is alinear and therefore a calibration curve must be constructed once for each standard gas. Curves for 4 convenient standard gases, in this case, 0.289, 0.110, 0.054, and 0.023 per cent CO (Fig. 1), have not changed in over 2 years even though analysis cells, windows, and amplifier tubes have been replaced. The amplifier section response is entirely linear (Fig. 2). Pure CO and standard CO mixtures can be obtained commercially.‡

Gas Analysis.—The amplifier section of the meter should be in operation for at least 8 hours prior to analyses; the pickup section requires little warm-up.

Gases can be introduced either by pressure from a gas tank or tonometer or by suction with a small pump; but all readings are made while the gas is not in motion and with the sample at atmospheric pressure. Only 125 ml. of gas are needed to flush the drying tube and sample chamber if it is introduced at a rate of about one-half liter per minute.

The zero reading is adjusted with dry room air, small amounts of CO in the air being of no concern provided air from the same room is used for dilution during Van Slyke extraction. The standard gas then is introduced and the gain adjusted until the meter reads 100. This procedure is repeated until both the "zero" and the "100" readings check twice.

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*Model 15 gas analyzer, fitted with 10 ¼-inch absorption cell, 5-inch filter cell filled with pure, dry carbon dioxide, 15 ¼-inch dummy cell, and sapphire windows. The Liston-Becker Division, Beckman Instruments, Inc., 649 Hope Street, Springdale, Conn.

†Model 273, 0 to 1 Ma. range, 0.25 per cent accuracy, 200 scale divisions; Weston Electrical Instrument Corp., Newark, N. J. For adequate damping the meter should be shunted with a 500 m.f.d. condensor.

‡The Matheson Company, Inc., East Rutherford, N. J.

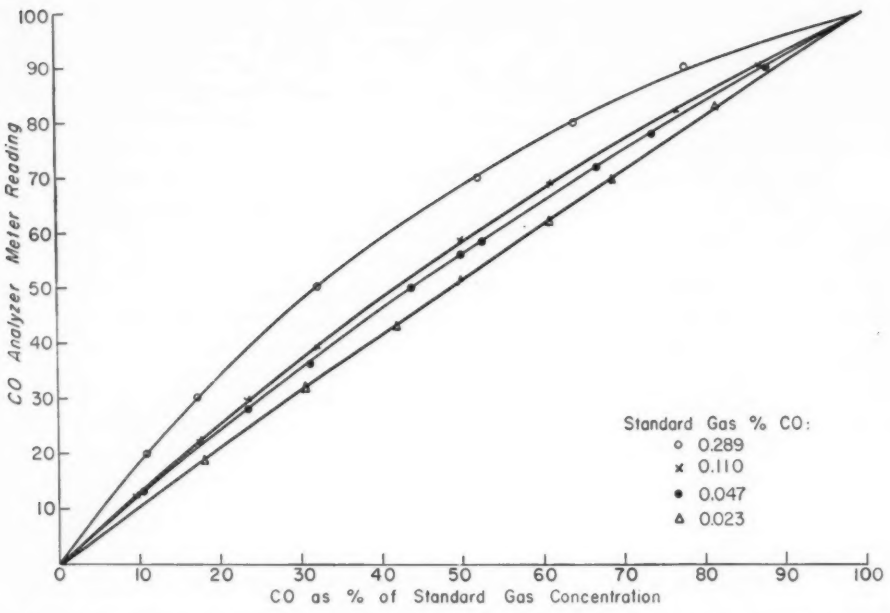

Fig. 1.—Typical calibration curves for various concentrations of standard gas. The meter reading for an unknown sample is converted to CO content as per cent of standard gas by reference to these curves.

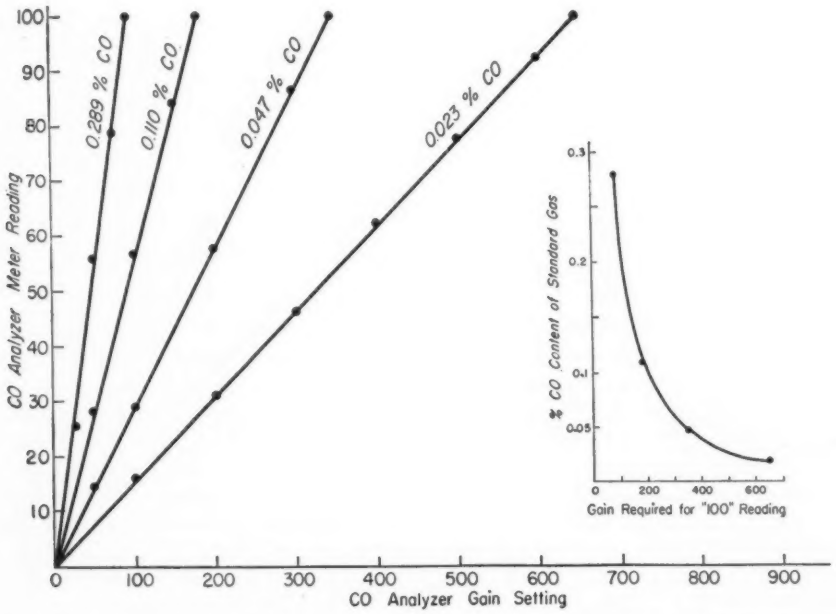

Fig. 2.—Meter deflections for various standard gases and with different gain settings. The amplifier section of the analyzer is entirely linear. The insert shows the gain setting required to obtain a full-scale meter deflection with the different standard gases.

The required gain adjustment for a "100" reading with various gases is shown in Fig. 2. The standardization procedure usually requires from 3 to 5 minutes and should be repeated every one-half hour. Gas samples can be analyzed at the rate of one every 2 to 3 minutes. Meter readings are converted to CO content as per cent of standard gas by reference to the calibration curve (Fig. 1).

Calculation.—The blood CO content, in ml./100 ml. (volumes per cent, STPD), is calculated from the volumes of blood and tonometer, and from the CO concentration of the gas sample. For example, with 3 ml. blood, a 150 ml. tonometer, and 0.020 per cent CO measured in the gas, the calculation is $150 \times 0.02 \div 3 = 1.00$ ml./100 ml., with appropriate corrections to STPD.[11]

The COHb saturation, in per cent, can be calculated from the CO content if the CO capacity of the blood is known. This capacity can be determined in several ways: (1) directly, by exposing the blood to 100 per cent CO and repeating the CO content determination after equilibration (with appropriate corrections for dissolved CO), (2) by measuring the oxygen capacity of the sample and adding the CO content, or (3) by spectrophotometric hemoglobin determination. These considerations were discussed in detail by Van Slyke and associates.[3]

RESULTS

Precision of the Method and Choice of Decomposing Solution.—All experiments for appraisal of accuracy of the method were made with 3 ml. blood samples, 150 or 300 ml. tonometers, and approximately 0.05 per cent standard gas.

Duplicate determinations, using ferricyanide as the decomposing solution, are shown in Table I arranged in two groups according to results below or above 1.0 volume per cent CO. The standard deviation of the variance of paired observations was 0.023 ml. per 100 ml. for both groups. The coefficient of variation for the entire series of 50 duplicates was 2.2 per cent. For serial analyses of 6 bloods, the S.D. of the variance for the series was 0.024 and the coefficient of variation was 1.5 per cent.

There is some controversy whether acid ferricyanide or sulfuric acid is preferable for decomposition of the blood prior to gas extraction.[6-8] With bloods of CO content of less than 2.5 ml. per 100 ml. the use of 5 per cent sulfuric acid led to a S.D. of the variance of ±0.015 and a coefficient variation of 1.5 per cent (Table I) which compared favorably with ferricyanide. Duplicate analyses of 11 bloods by each method showed means and S.D. identical for both groups and the "t" test revealed no significant difference between methods (Table II). However, with bloods containing more than 2.5 ml. per 100 ml. CO there were sudden large discrepancies between means (Table I) and the appearance of the material in the Van Slyke chamber suggested that not all blood had been converted to methemoglobin. This gumming-up of unchanged blood could not be prevented by increasing the sulfuric acid concentration up to 35 per cent. At present, we prefer sulfuric acid for low concentrations of CO because cleaning of the Van Slyke chamber is a great deal easier. For high concentrations and for CO capacity determinations, acid ferricyanide must be used.

TABLE I. PRECISION OF CO CONTENT DETERMINATION
(METHOD: 3 ML. SAMPLE, 150 TO 300 ML. TONOMETER, 0.054 PER CENT CO STANDARD GAS)
(ALL VALUES: CO CONTENT IN ML. PER 100 ML.)

	ACID FERRICYANIDE						SULFURIC ACID	
	DUPLICATE DETERMINATIONS				SERIAL DETERMINATIONS		DUPLICATE DETERMINATIONS	
	CO CONTENT < 1.0		CO CONTENT > 1.0				CO CONTENT > 2.5	
					SERIES	MEAN		
	0.73	0.73	1.78	1.76	1.28		3.18	3.06
	0.81	0.76	1.28	1.36	1.25		4.62	4.36
	0.70	0.70	1.67	1.67	1.24		5.01	5.32
	0.70	0.66	1.08	1.10	1.25	1.26	2.89	3.01
	0.18	0.17	1.84	1.84	1.28		4.12	4.42
	0.17	0.17	1.12	1.06	1.26		2.86	2.97
	0.78	0.81	1.53	1.58	1.28	1.27		
	0.72	0.84	1.77	1.76	2.10		CO CONTENT < 2.5	
	0.14	0.16	1.13	1.14	2.14			
	0.12	0.11	1.51	1.57	2.16			
	0.11	0.09	1.23	1.23	2.18		0.44	0.43
	0.73	0.75	1.67	1.69	2.19		0.57	0.57
	0.96	0.97	1.43	1.39	2.10		0.98	0.98
	0.69	0.70	1.23	1.22	2.16		0.09	0.10
	0.24	0.24	1.13	1.13	2.13	2.14	0.65	0.67
	0.69	0.67	1.06	1.06	2.27		1.25	1.27
	0.65	0.65	1.37	1.40	2.28		1.51	1.50
	0.67	0.67	1.25	1.26	2.26	2.27	1.09	1.12
	0.63	0.65	2.46	2.53	0.82		1.37	1.38
	0.86	0.86	3.56	3.59	0.83		1.28	1.28
	0.49	0.49	3.66	3.65	0.83	0.83	1.14	1.14
	0.55	0.55	3.29	3.30	1.71		1.21	1.15
	0.16	0.15	4.49	4.49	1.72		1.12	1.12
	0.11	0.11	4.60	4.55	1.72	1.72	1.05	1.06
	0.92	0.99	4.41	4.42			2.46	2.47
Mean	0.544		2.065		1.602		0.983	
S.D. of Variance	±0.023		±0.023		±0.024		±0.015	
Coeff. of Variation	4.2 per cent		1.1 per cent		1.5 per cent		1.5 per cent	

TABLE II. COMPARISON OF DIFFERENT DECOMPOSING SOLUTIONS AND NUMBER OF EXTRACTIONS
(ALL VALUES AS CO CONTENT IN ML. PER 100 ML. AND ALL VALUES ARE MEANS OF DUPLICATE DETERMINATIONS)

			ACID FERRICYANIDE				
	ACID FERRICYANIDE	5 PER CENT SULFURIC ACID	MULTIPLE EXTRACTIONS MEANS	SINGLE EXTRACTION		DOUBLE EXTRACTIONS	
				MEANS	RESIDUAL GAS	MEANS	RESIDUAL GAS
	1.55	1.51	1.77	1.72	trace	—	–
	1.23	1.26	0.64	0.58	0.04	—	–
	0.49	0.44	1.22	1.11	0.06	—	–
	1.13	1.11	1.84	—	—	1.87	0
	1.06	1.03	1.56	—	—	1.58	0
	0.56	0.57	0.18	—	—	0.17	0
	1.39	1.38	0.80	—	—	0.80	0
	1.26	1.28	0.12	—	—	0.11	0
	1.08	1.14	2.07	—	—	2.07	trace
	2.28	2.29					
	0.13	0.10					
Mean	1.103	1.098					
S.D.	±0.58	±0.58					
S.E.	0.17	0.17					
t	0.021						

Number of Vacuum Extractions.—Repeated shaking and evacuation to fill the tonometer is quite time consuming. Therefore a study was made to ascertain the minimum number of necessary vacuum-shaking periods. The CO content calculated after a *single* vacuum extraction of a given blood was compared to that after enough extractions to fill the tonometer. Usually the content after a single extraction was 0.04 to 0.11 volume per cent lower than the content after multiple extractions and the gas obtained after a further shaking period still contained demonstrable amounts of CO (Table II). After *two* extractions the CO content was the same as after multiple extractions and the residual gas no longer contained traces of CO. The adequacy of the double extraction method, further confirmed by a number of quadruplicate analyses (Table II), was adopted as routine.

Blank Determinations.—Six duplicate analyses were made using the usual 4 ml. acid ferricyanide, but with 3 ml. of distilled water instead of blood; a similar study was made with sulfuric acid. On five occasions there was no CO indication on the meter and once there was a slight movement of the needle, but less than 0.2 per cent of full scale. A blank correction was therefore unnecessary. Lawther and Apthorp[10] found blank readings up to 0.11 volume per cent in spite of meter calibration and gas dilution with CO-free air. Consequently, we repeated blank determinations using their ferricyanide-saponin solution. Blank CO values were then obtained approaching, and even exceeding, theirs, depending upon the brand of saponin. It was concluded that saponin gives off small quantities of CO.

Comparison With Conventional Methods for COHb Determinations.—The method of Scholander and Roughton,[4] recommended for blood containing small quantities of CO, was used in our laboratory before the present method was developed. Absorption of oxygen prior to measurement of carbon monoxide presented a problem because the conventional sodium hydrosulfite solution also absorbs small amounts of CO, while pyrogallol evolves traces of CO. This problem, the difficulty of the syringe technique, and certain vagaries of the results were responsible for our search for a new type of analysis. Results of repeated tests of 3 bloods by our method and that of Scholander and Roughton are shown in Table III. The variability with the syringe method should not be considered a reflection of this technique but merely demonstrates our lack of experience with it. The data are sufficient to show at least that the same measurement is made by both techniques.

A few parallel determinations were made by our technique and that of Van Slyke and associates[3] (Table III). The considerable variability of repeated tests by this method was due to constantly changing and large blank corrections in spite of elaborate precautions to prevent introduction of inert gases with the reagents, vacuum-pump extraction of all solutions, and storage under air-free oil. Again, the range of the results by the two methods was of the same order.

The Effect of Cold Storage of Blood.—Duplicate determinations were made of the same blood immediately after collection and after storage on ice for

24 hours. In vitro formation of CO, which has been reported to occur during incubation of blood at 37° C.,[12] could not be demonstrated at these low temperatures (Table III).

TABLE III. COMPARISON WITH OTHER METHODS FOR CO ANALYSIS
(ALL VALUES: CO CONTENT IN ML. PER 100 ML.)

SAMPLE NO.	PRESENT METHOD	SCHOLANDER AND ROUGHTON[4]	PRESENT METHOD	VAN SLYKE AND ASSOCIATES[5]
1	0.46	0.86	0.82	0.68
	0.47	0.44	0.83	0.09
	0.47	0.44	0.83	1.09
2	0.17	0.27	1.71	1.87
	0.17	0.18	1.72	1.92
			1.72	1.96
3	5.80	5.50	2.48	2.17
	5.83	5.76	2.50	2.21
4			1.38	1.33
			1.39	1.32
Mean	1.91	1.92	1.54	1.46

EFFECT ON CO CONTENT OF COLD STORAGE AND OF EXPOSURE TO AIR
(ALL VALUES: CO CONTENT IN ML. PER 100 ML. AND MEAN OF DUPLICATE DETERMINATIONS)

SAMPLE NO.	IMMEDIATE ANALYSIS MEAN	AFTER 24-HOUR STORAGE ON ICE MEAN	IMMEDIATE ANALYSIS MEAN	AFTER ROTATION IN AIR	
				MEAN	MINUTES OF ROTATION
1	1.69	1.67	1.09	1.02	4
2	2.49	2.50	1.09	1.07	6
3	0.89	0.90	1.84	1.75	4
4	0.79	0.79	0.70	0.68	10
5			0.18	0.17	10
6				0.78	10
Mean	1.56	1.56	0.95	0.91	

Effect of Exposure of Blood to Air.—The question was raised whether the blood CO content decreases measurably during prolonged exposure to air required for the customary oxygen capacity determination. Six samples of arterial blood were obtained with anaerobic precautions and a portion was analyzed immediately in duplicate. Blood samples of 2.5 ml. then were delivered into several open, wide-neck, 35 ml. bottles and rotated on a motor board for periods varying from 4 to 10 minutes. Analysis for CO content then proceeded in the usual manner. The exposed bloods always contained less CO but often the diminution was within the limit of error of the technique (Table III). It was concluded that: (a) a small air bubble accidentally aspirated into the syringe during sampling should not alter significantly the result of a routine CO content analysis, and (b) exposure to air for capacity measurements may cause a significant error if CO content and O_2 capacity are to be added for computation of total active hemoglobin.

Applications of the Method.—

1. *The "normal" carboxyhemoglobin saturation:* Human blood apparently always contains minute quantities of carboxyhemoglobin. In our series of nonsmokers, drawn from an urban population, the blood CO content ranged

from a low of 0.10 to a high of 0.24 ml. per 100 ml., corresponding to COHb saturations of 0.62 and 1.24 per cent, respectively (Fig. 3). In smokers, the COHb saturation may reach rather high values.[13] The blood COHb saturations shown in Fig. 3 were plotted without regard to intensity of smoking or time of sampling; yet, the mean of 4.67 per cent for 9 smokers was almost six times greater than the mean of 0.85 per cent of 9 nonsmokers. Analysis of this kind may offer an opportunity for objective and quantitative assessment of smoking habits.

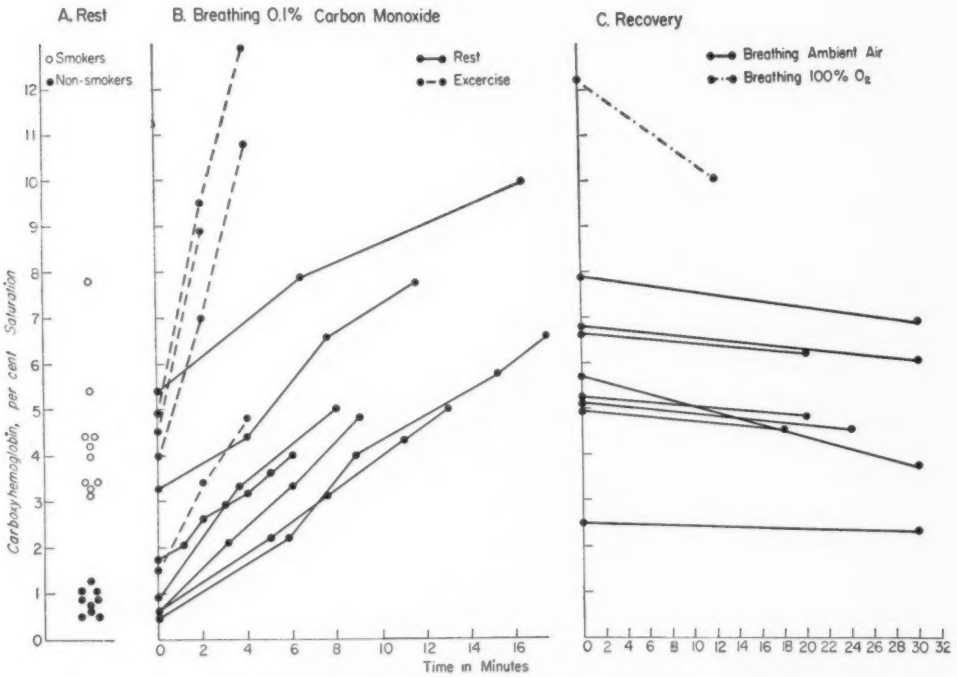

Fig. 3.—*A*, Arterial carboxyhemoglobin saturations of smoking and nonsmoking city dwellers. *B*, Increase of COHb saturation during inhalation of 0.1 per cent CO in air at rest and during moderate treadmill exercise. *C*, Decrease of COHb saturation with room air or oxygen breathing following inhalation of 0.1 per cent CO.

2. *The COHb saturation during CO breathing and recovery:* Our interest in the rate of blood CO increase during CO breathing was stimulated by an attempt to estimate the CO "back pressure" from the pulmonary capillary blood during lung diffusing capacity measurements.[14] Arterial COHb saturations, obtained before and at intervals during 0.1 per cent CO breathing, are shown in Fig. 3. At rest, the rate of increase was practically the same for all subjects, about 0.33 per cent per minute, which corresponds quite well to previous estimates of fatal outcome from breathing such a mixture for 1.5 to 3 hours. During mild exercise on a treadmill (8 per cent incline, 3 m.p.h.), the rate of CO uptake was about six times greater than at rest and a rough parallel existed between this increase and that of oxygen consumption, ventilation, and cardiac output.

The rate of recovery during room air breathing was slow (Fig. 3) and roughly proportional to the initial level of saturation. Although logarithmic decay was not demonstrated, the data bear out previous estimates of a "half-life" of COHb of about 4 hours.[15] As is well known, the speed of recovery is greatly increased by oxygen breathing (Fig. 3).

3. *In vitro equilibration of blood with CO:* Information concerning the carbon monoxide-hemoglobin dissociation curve is fragmentary, in part, because of the slowness of the CO-Hb reaction and the much lesser efficiency of tonometric systems compared to that of the lungs illustrated in Fig. 4. Whole human blood was agitated in a Kugeltonometer[16] at 37° C. while being exposed to a stream of humidified CO mixture. Both with 0.1 and 0.025 CO, the COHb saturation increased in linear fashion and even at the end of 4.5 hours there was no tendency toward equilibration.

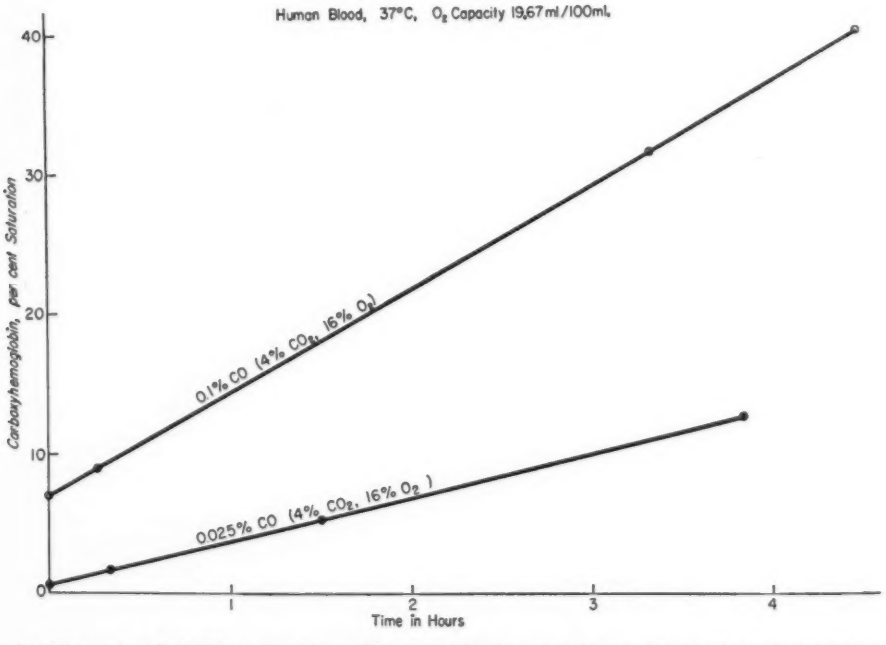

Fig. 4.—The rate of COHb saturation of human blood exposed to a constant stream of humidified CO (0.1 and 0.025 per cent) in a rotating Kugeltonometer at 37° C.

4. *Blood volume determination:* The laborious modification of the original Gréhant-Quinquaud blood volume method, proposed by Root, Roughton, and Gregerson,[15] appears to be the technique with claim to highest precision. Insistence on determination of CO content before the test is pertinent because neglect of this factor, particularly in smokers, may cause an error of as much as 25 per cent (Fig. 3). The open circuit breathing system avoids certain systematic errors and permits an exact allowance for the loss of CO from the blood by all slow routes, whatever their nature. The present method facilitates rapid CO determinations of both blood and gas, and easily analyzed com-

mercial CO mixtures eliminate the need for tedious CO manufacture and volumetric dilution. During a comparison of simultaneously obtained blood volume by this and the conventional T-1824 method, all analytical work was completed in 20 minutes.

The CO blood volume method, with the present modifications, is quite simple and very precise and, after some years of neglect, deserves reconsideration.

DISCUSSION

Selectivity of the CO Analyzer.—The analyzer can be sensitized to all gases which have absorption bands in the infrared spectrum.[9] These bands are caused by vibration of molecular bonds and, hence, the meter can respond only to heteroatomic gases and is entirely unaffected by N_2 and O_2 in the sample. A high degree of selectivity for CO is obtained by the CO-filled detection chambers. However, there is a very slight response to water vapor and CO_2. Moisture is removed by the drying tube; the small response to CO_2 is minimized by an "interference" cell charged with dry CO_2, but, due to overlapping absorption bands, cannot be entirely eliminated.

Estimation of the Error Due to the Presence of CO_2 in the Sample.—This can be estimated by assuming a normal blood CO_2 content of 50 ml. per 100 ml. Then, with a 3 ml. blood sample and a 300 ml. tonometer, the CO_2 concentration in the tonometer would be 0.5 per cent. A gas containing 0.5 per cent CO_2 in air at full gain leads to a meter reading of 0.5 per cent of full scale, corresponding to an equivalent CO response of 0.000125 per cent; the meter is thus 4,000 times more sensitive for CO than for CO_2. The "false" CO content in the blood sample under discussion would then be only $0.000125 \times 300 \div 3$ or 0.01 ml. per 100 ml. Even with respiratory acidosis and the smallest tonometer the error could never exceed 0.04 ml. per 100 ml. However, for most precise work an appropriate correction could be made by prior CO_2 analysis of the gas or blood sample.

Sixteen analyses were performed to test the validity of these assumptions. Duplicate tests of CO content were made using 3 ml. blood samples, 150 ml. tonometers, and (1) the acid ferricyanide solution alone or (2) this solution plus 1 ml. 1 N KOH for absorption of the CO_2 evolved during extraction (Table I). There was no consistent difference in results by the two techniques and the means for each group were both 0.90 ml. per 100 ml. CO.

Theoretical Accuracy of the Method and Choice of Sample Size, Tonometer Volume, and Standard Gas.—The modified CO meter can be read to 0.2 per cent of full scale. With the lowest standard gas (0.025 per cent CO), 0.2 scale divisions correspond one-half part per million or 0.00005 per cent CO; with a 150 ml. tonometer and a 3 ml. blood sample this should make it possible to read $0.00005 \times 150 \div 3 = 0.0025$ ml. per 100 ml. blood CO content and concentrations up to 1.25 ml. per 100 ml. could be read with this standard. Since higher values are often encountered in experimental procedures, particularly in smokers (Fig. 3), the sensitivity of the meter must be reduced or higher standards used. We have found it best to use approximately 0.05 per cent

CO as the routine standard gas and to change from 150 to 300 ml. tonometers, as required. Then, the maximal theoretical accuracy is 0.005 to 0.01 ml. per 100 ml., which is still three to ten times greater than that claimed for any other method. With 0.1 per cent CO as standard gas, a 1 ml. sample of blood, fully saturated with CO, can be read with an error of 0.06 volume per cent.

These calculations apply only to the relative accuracy of the method. A precise definition of blood CO content in absolute terms is not required when pressure differences or rate of concentration changes are considered as for blood volume or pulmonary diffusion measurements. If the blood CO content must be known in absolute terms, the accuracy of results will depend on the quality of the standard CO mixture. Commercially prepared dilutions of CO in our experience correspond to the labeled concentration to no better than ± 10 per cent. Furthermore, the CO content of compressed gas may change over the course of a few months due to further mixing of gases or oxidation of CO catalyzed by rust and other impurities. For calibration of standards, gases of accurately known CO composition can be prepared by mixing a known volume of pure (98.6 per cent) CO* from a tonometer with a known volume of gas freed of CO by Hopcalite† from a Tissot spirometer. CO mixtures analyzed or mixed with a high degree of accuracy can also be obtained from the manufacturer of the infrared analyzer, from commercial testing laboratories, or from governmental agencies.

SUMMARY

A simple, rapid, highly accurate method is described for determination of carbon monoxide content of blood. The chamber of the manometric Van Slyke apparatus is used for decomposition and vacuum extraction. The gas is diluted in a tonometer and analyzed for CO content with an infrared meter of great specificity and good stability, which has been modified for highest accuracy.

Theoretically, readings to 0.0025 ml. per 100 ml. CO content are possible with a 3 ml. blood sample. In practice, the S.D. of the variance of 50 paired observations was 0.023 ml. per 100 ml. and the coefficient of variation was 2.1 per cent.

The error due to the presence of CO_2 in the extracted gas was shown to be within the limit of accuracy of the technique. At low concentrations of CO, results were the same whether acid ferricyanide or sulfuric acid was used for conversion to methemoglobin; with higher CO concentrations, sulfuric acid led to incomplete decomposition. Saponin should not be used because it contains traces of CO. Blank determinations were shown to be unnecessary. Cold storage of blood up to 24 hours and slight exposure to air of the sample did not affect the results.

Results by this method were compared to those obtained by more conventional techniques.

*The Matheson Company, Inc., East Rutherford, N. J.

†Active Hopcalite, Mine Safety Appliances Company, 201 North Braddock Avenue, Pittsburgh 8, Pa.

A number of applications are illustrated including assessment of smoking habits, the COHb saturation during CO breathing, the CO-Hb equilibration time and dissociation curve, and determination of "true blood volume."

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A MICRODETERMINATION OF CALCIUM IN BLOOD SERUM

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SINCE Kramer and Tisdall[1] applied the classic technique of calcium oxalate precipitation and permanganate titration to serum, many methods have been devised for calcium determination in blood serum. The recent review of Leifheit[2] discusses a wide range of techniques and procedures for serum analysis, consisting of colorimetric, complexiметric, and flame photometric methods. The fact that there are so many calcium procedures indicates that a more rapid and accurate method is still needed.

Until recently, calcium determinations have required extensive treatment of the sample to remove interfering materials. Schwarzenbach and Gysling[3] have prepared an indicator and developed a method that would allow the direct titration of a sample containing calcium. The indicator, Murexide (ammonium purpurate), was later reported as being unstable[4] and of yielding vague and false end points when large amounts of magnesium were present.[5]

Diehl and Ellingboe[5] recently published a method which allows the direct titration of calcium in inorganic materials in the presence of magnesium. The indicator used was Calcein, which is prepared by condensing iminodiacetic acid with fluorescein. In the method here proposed, their technique has been modified, adapted to a microscale, and successfully applied to the analysis of blood serum.

In the proposed method, a given amount of disodium dihydrogen ethylenediamine tetraacetate (EDTA) is added to the serum sample sufficient to complex all of the calcium and magnesium present and to provide an excess. Any iron or copper that would otherwise interfere is then complexed by the addition of sodium cyanide. The indicator Calcein is added and the pH adjusted to 12 or above. In the presence of excess EDTA, the indicator is not complexed with calcium at this pH, and has a dull brown color. If the pH is below 12, the indicator has a bright yellow-green color, whether excess calcium is present or not. A standard solution of calcium is added to the EDTA-serum solution, complexing the excess EDTA and displacing the magnesium from the magnesium EDTA complex. When an excess of calcium is added, the indicator becomes bright yellow-green, and fluoresces under ultraviolet light.

The procedure is rapid, requires only 0.2 ml. of serum, and its error is ±3 per cent. No treatment of the sample is necessary and no graphs or double titrations are used.

From the Research Laboratories, Don Baxter, Inc., Glendale, Calif.
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MATERIALS AND REAGENTS

1. *Misco microburet:* 0.1 ml. capacity.*
2. *Blak-Ray ultraviolet lamp:* (Long wave).†
3. *Test tubes:* Approximately 12 × 100 mm.
4. *Compressed air bubbler:* For mixing during titration.
5. *EDTA solution:* Prepare approximately 0.002 M EDTA by dissolving 0.75 Gm. EDTA‡ in 1 L. of water.
6. *Standard calcium solution:* Prepare 100 mg. per cent calcium by dissolving 2.4973 Gm. calcium carbonate (dried reagent grade) in 15 ml. 2 N HCl and diluting to 1 L. with freshly boiled and cooled distilled water.
7. *Stock Calcein solution:* Dissolve 0.25 Gm. Calcein powder§ in 4 ml. 1 N NaOH and add 30 ml. of water.
8. *Calcein indicator solution:* Dilute 1 ml. of stock Calcein solution with water to 100 ml. (This dilute solution gradually loses strength and should be made fresh every 3 days.)
9. *One N NaOH:* Dissolve 4 Gm. solid reagent NaOH in 100 ml. of water.
10. *NaCN solution:* Dissolve 1 Gm. NaCN in 100 ml. of water.
11. *Anti-foam suspension:* Mix 0.1 Gm. Dow-Corning Anti-foam‖ with 100 ml. of water.

PROCEDURE

Pipette into a test tube:

0.20 ml. serum
1.00 ml. EDTA solution

add:

1 ml. Calcein indicator solution
3 drops each of:

NaCN solution
1 N NaOH
Anti-foam solution

Titrate with standard calcium solution, using the ultraviolet lamp placed about 3 inches above the sample as the only artificial illumination. The equivalence of the EDTA solution is determined by titrating 1.00 ml. EDTA solution with standard calcium solution, using all the reagents that are used in the serum titration. This standardizes the EDTA solution and provides the necessary end point corrections.

Calculations.—Milligram per cent calcium = (volume standard calcium solution to titrate 1 ml. EDTA solution minus volume standard calcium solution to titrate serum sample) × 500.

RESULTS

Inorganic Standard Solutions.—Solutions approximating the calcium and magnesium content of rabbit serum were prepared and analyzed to determine the effect of magnesium. The results in Table I demonstrate that magnesium has no effect on the calcium determination.

Serum.—Fresh and ashed samples of pooled rabbit serum were analyzed to determine the effect of organic constituents of serum on the determination. The results in Table II indicate the precision of the method and show a close

*Microchemical Specialties Company, Berkeley, Calif.
†Ultra-Violet Products, Incorporated, South Pasadena, Calif.
‡Sequestrene Na_2, Alrose Chemical Company, Providence, R. I.
§G. Frederick Smith Chemical Company, Columbus, Ohio.
‖Dow-Corning Corporation, Midland, Mich.

agreement between duplicate samples, ashed and fresh, of the same serum. Thus, the organic constituents of serum have no effect on the calcium determination.

TABLE I. DETERMINATION OF CALCIUM IN INORGANIC STANDARD SOLUTIONS

CONCENTRATION OF CALCIUM (MILLIGRAM PER CENT)*		
ACTUAL	FOUND BY ANALYSIS	PER CENT ERROR
13.0	12.7	−2.5
13.0	12.7	−2.5
13.0	12.7	−2.5
13.0	13.2	+1.5
12.0	12.4	+3.5
12.0	12.3	+2.5
12.0	12.2	+1.5
10.0	9.95	−0.5
Mean 12.3	12.3	Average error ±2.1 per cent

*Magnesium content approximately 7 mg. per cent.

TABLE II. ANALYSIS OF POOLED RABBIT SERUM

FRESH	ASHED
11.9	11.6
11.8	11.5
10.5	11.4
11.5	11.7
11.4	11.3
Mean 11.4 ± 0.2 mg. per cent	11.5 ± 0.1 mg. per cent

Standard Serum.—Two standard serum samples,* previously analyzed by the Clark-Collip[6] method, were analyzed by the above procedure. The data in Table III show that the proposed method compares favorably with the Clark-Collip[6] method.

TABLE III. ANALYSIS OF TWO CONTROL SERUMS

CONTROL SERUM A (CALCIUM = 10.2 ± 0.4 MG. PER CENT)	CONTROL SERUM B (CALCIUM = 9.9 ± 0.3 MG. PER CENT)
10.4	9.9
10.5	9.7
10.3	9.7
10.7	9.7
10.5	10.1
10.3	10.1
Mean 10.5 ± 0.1 mg. per cent	9.9 ± 0.2 mg. per cent

DISCUSSION

With some experience, the true end point of the titration is easily detected. As the equivalence point is approached, however, false end points will appear and subsequently fade until the true end point is reached. The fading is due to small excesses of calcium, which disappear as the magnesium-EDTA complex is broken. If most of the titrant (calcium solution) is added rapidly, the number of false end points is decreased and the titration is more rapid.

*Lab-Trol, Dade Reagents, Incorporated, Miami, Fla., and Clinical Chemistry Control Serum, Hyland Laboratories, Los Angeles, Calif.

Hemolyzed serum, due to its red color, causes difficulty in seeing the end point and practice is necessary to obtain accurate results. The addition of cyanide complexes most of the interfering metallic ions of hemolyzed serum.

The method has been applied to urine and should give accurate results with light-colored or colorless body fluids. Larger volumes of solution and larger quantities of calcium than 0.2 ml. and 0.04 mg. of calcium can be determined by this method, if desired. The amounts of the various reagents should be increased in proportion to the increased calcium being assayed.

SUMMARY

1. A simple titrimetric method for the rapid determination of calcium in 0.2 ml. of serum is presented.

2. The main advantage of the method is that the determination is performed directly on the sample, and requires no precipitations or special treatment.

3. Data are given to show satisfactory precision and accuracy for inorganic standard solutions, control serum, and blood serum.

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THE MEASUREMENT OF AMMONIA IN WHOLE BLOOD, ERYTHROCYTES, AND PLASMA

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REVIEWS of the literature by Conway[1] and Kirk[2] indicate that existing methods for measuring free ammonia in blood yield values ranging from 0 to 3 mcg. of ammonia-nitrogen per milliliter of human blood. These methods depend upon alkalinization of blood followed by isolation of its ammonia using aeration,[2-4] distillation,[5, 6] or diffusion.[7] Conway,[1, 7] using his microdiffusion technique, made the blood alkaline with potassium carbonate (K_2CO_3) and measured the diffused ammonia by titration. He reported that no free ammonia was present in freshly drawn human venous blood. However, when allowed to stand exposed to air so that dissolved CO_2 was lost from the blood, the ammonia content rose in 5 minutes to about 0.46 mcg. of N per milliliter. Recently White and associates[8] have verified Conway's observation in human blood using the latter's analytical procedure. Koprowski and Uninski,[9] using the same method, obtained similar data with dog blood. Other investigators (cited by Conway[1] and Kirk[2]) have found ammonia to be present in freshly drawn blood.

The method described in this paper demonstrates the presence of free ammonia in blood. Our studies reveal artifacts which may produce low or high values because of (1) slowing of diffusion by blood proteins, (2) occasional incomplete recovery of ammonia from blood made alkaline with K_2CO_3, and (3) hydrolysis of labile compounds which yield ammonia. The method is based on the conversion of ammonium ions to ammonia gas by alkalinization with a buffered potassium carbonate-bicarbonate mixture, followed by isolation of the ammonia using the diffusion system of Seligson and Seligson,[10] and measurement of the ammonia colorimetrically after treatment with Nessler reagent.[11]

APPARATUS

1. Diffusion bottles, 50 ml. (43 × 73 mm. with a 13 mm. inlet, No. s-18B, sold by T. C. Wheaton, Millville, N. J.), see Fig. 1.

2. Receiving bottles, 10 ml. (25 × 53 mm. with a 13 mm. inlet, No. s-407, sold by T. C. Wheaton Company).

3. Rubber stoppers to fit the above bottles (corkage B, s-46 plug, sold by T. C. Wheaton Company).

4. Receiving rods (Fig. 1.), 6 × 80 mm. glass rod with a constriction 12 mm. from one end. The short end below the constriction is ground so that when dipped into acid it retains an acid film over its surface. The rods are fitted into the bored rubber stoppers, described above, as shown in Fig. 1. This combination, when fitted into the 50 ml. bottle, will allow the rod to extend halfway into the bottle and at the same time seal it.

5. Stainless steel rods approximately 8 mm. diameter × 40 mm. long.

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6. Rotator, Fig. 2. This device has a wheel to which spring clamps are attached to hold the diffusion bottles. The wheel rotates at 50 revolutions per minute. The spring clamps may be so placed as to hold bottles of other dimensions for the various determinations[10] involving measurement of ammonia. The bottles are placed 8 cm. from the center of the wheel to prevent the stainless steel rods within from whipping around and splashing blood on the receiving rod.

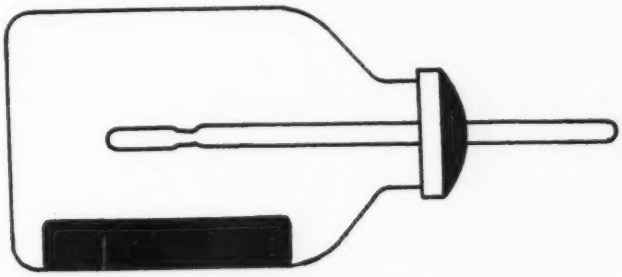

Fig. 1.—Bottle for NH_3 diffusion containing a receiving rod for collecting diffusate and a stainless steel rod for facilitating diffusion and mixing.

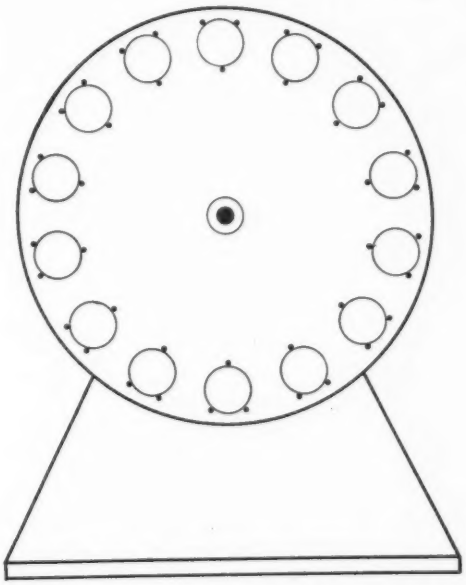

Fig. 2.—Rotator for diffusion bottles. Three pins hold each bottle on a wheel which rotates 50 r.p.m. Bottles are placed 8 cm. from turning shaft.

7. Spoon to deliver approximately 3.0 Gm. of alkali mixture described below.

8. A photometer. The studies herein reported were made with a Beckman Model DU Spectrophotometer using a 10 mm. cell at a wave length setting of 395 mμ. Analyses for physiologic studies have been made on a simple photometer using a filter absorbing at 415 mμ and an 18 mm. test tube cuvette which required a minimum of 4 ml. of reaction mixture. Many other instruments should serve the purpose.

REAGENTS

1. Alkali mixture, 2 parts of $K_2CO_3 \cdot 1\frac{1}{2}\ H_2O$ plus 1 part $KHCO_3$ mixed thoroughly but not powdered. Dry or partially dry K_2CO_3 will cause the blood to clump and become warm.

If foaming occurs on mixing with blood, suspend 0.5 Gm. Antifoam A (Dow Corning Corporation, Midland, Mich.) in 5 ml. of ether and triturate with 300 Gm. of salt mixture. Allow the ether to evaporate in the open room. To keep the salts moist, store in a closed container over saturated K_2CO_3.

2. 1 N H_2SO_4, 1 part concentrated H_2SO_4 plus 35 parts of water.

3. Nessler reagent of Vanselow.[11] (Dissolve 45.5 Gm. HgI_2 plus 34.9 Gm. KI in as little water as possible. Add 112 Gm. KOH in 140 ml. water, dilute to 1 L. Let stand 3 days.) Dilute 1:20 before use.

4. Standard $(NH_4)_2SO_4$, 0.300 mg. NH_3-N per ml. or 1.415 Gm. $(NH_4)_2SO_4$ per liter. The working standard of 3.00 meg. per ml. is made on the day of use by diluting the standard 1:100.

5. Water. Single distilled water passed through a sulfonic acid ion exchange resin in the hydrogen cycle (I R 120—Rohm and Haas) is suitable.

PROCEDURE

Three stainless steel rods and a spoonful of carbonate-bicarbonate salt mixture (3.0 Gm.) are loaded into each bottle. These are placed serially on their sides in preparation for use. Two milliliters of blood are pipetted into a bottle and immediately capped with a stopper. The carbonate salts and blood are allowed to mix on the rotator for one minute, and then a receiving rod, wetted by dipping it into 1 N H_2SO_4 to the indentation, is inserted into the bottle replacing the stopper (Fig. 1). At this stage the bottle may be placed aside until 8 or 10 such bottles have been prepared in rapid succession. Two blanks (2 ml. water) and 2 standards (2 ml. of working standard) are prepared first, 2 or 3 samples in duplicate are prepared last. Then all the bottles are placed on the wheel of the rotator which is turned at 50 r.p.m. for 20 minutes. The diffused ammonia is received by the acid on the glass rod. The receiving rods are transferred to clean receiving bottles, the necks of which fit the attached rubber stopper. At a convenient time the ammonia is washed from the rod into the vessel with 3 ml. of the diluted Nessler reagent. After 5 minutes the optical density of the yellow-orange solution is measured in a 10 mm. cell at 395 mμ in a Beckman spectrophotometer using the Nessler reagent as the reference. If a photoelectric colorimeter is used, add 4 ml. and read at 415 mμ, or as close to the preferred setting of 395 mμ as possible. The optical density (O.D.) readings are corrected for the reagent blank and the calculations are made from the standard as follows:

$$\text{Micrograms } NH_3\text{-N per ml. blood} = \frac{3.00}{\text{O.D. of Standard}} \times \text{O.D. of Unknown.}$$

DISCUSSION OF THE TECHNIQUE

The large bottle is used as a diffusion vessel because it allows mixing of the akalinized blood without contamination of the receiving rod. The stainless steel rods aid in completely mixing the carbonate salts with blood while simultaneously increasing the blood surface from which the ammonia diffuses. The rate of diffusion is thereby increased. Preliminary mixing of blood for one minute assures prompt solution of the salts in blood and prevents excessive rise of pH because it facilitates the solution of the less soluble bicarbonate simultaneously with the more soluble carbonate.

In preparation, the bottles with stainless steel rods in place are cleaned by rinsing several times with tap water and then once with ammonia-free water. They are dried in an oven at 120° C. After cooling, 3 Gm. of carbonate-bicarbonate mixture are added. Each bottle is capped with a rubber plug and then stored until used. This prevents contamination of the bottles with ammonia from the air. The glass receiving rods are rinsed with distilled water before use and dried in the oven. In this manner, trace contamination of ammonia is easily avoided. The method can be performed in an open laboratory because most of the procedure occurs in a closed system. Marked variations of room temperature should be avoided because rates of diffusion and hydrolysis are affected by temperature.

The reagents used are of the analytical grade and need no purification. Blanks of the system ranged from 2 to 5 per cent of the ammonia found in normal bloods depending upon the quality of the purchased reagents.

The mixture of 1 Gm. of $KHCO_3$ and 2 Gm. $K_2CO_3 \cdot 1\frac{1}{2}\ H_2O$ essentially forms a saturated solution with 2 ml. of blood. Addition of $KHCO_3$ to a blood containing K_2CO_3 maintains the pH at 11.0 to 11.5. With the omission of bicarbonate, the pH of the blood-K_2CO_3 mixture rises to 12.5 to 13.0. The reaction mixture of lower pH has a decided advantage which will be discussed below. Hydrated K_2CO_3 is more desirable than the anhydrous form because it does not give off heat while dissolving. The latter does, and, in addition, tends to cake the blood. Potassium salts are used because they do not inhibit diffusion of ammonia.[1] The anti-foam agent mixed into the salts prevents foaming which could contaminate the receiving rod.

The use of the Nessler reaction for determining ammonia in the diffusate proved to be more than adequate in terms of ease of use, reliability, sensitivity, and stability of reagent. The measurements in these experiments were made at 395 mμ because the ammonia-Nessler-reagent complex originally used in these experiments absorbed maximally at this wave length, although the maximum may vary with new reagents. The ammonia liberated from 2 ml. of normal blood gave optical density readings of the order of 0.150 at 395 mμ in a 1 cm. cell. The range of the standard curve was from 0 to 7 mcg. per ml. of sample, over which range optical density was proportional to concentration.

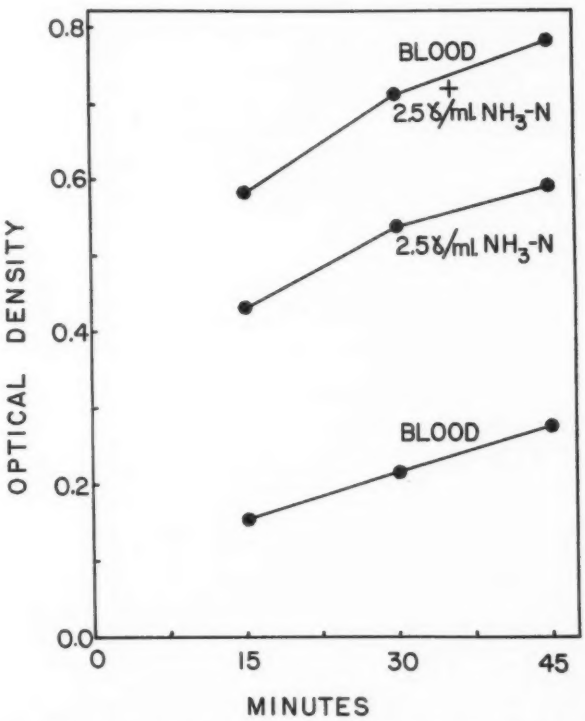

Fig. 3.—The effect of $K_2CO_3 \cdot 1\frac{1}{2}\ H_2O$ plus $KHCO_3$ as an alkalinizing agent to facilitate diffusion of ammonia from fresh blood; the same blood plus 2.5 mcg. NH_3-N per ml. and water containing 2.5 mcg. NH_3-N per ml. The abscissa represents the time of diffusion.

RESULTS

Study of the Method.—Fig. 3 shows the type of time-diffusion curve obtained with the method described above. Table I gives similar data obtained

from 4 samples of blood. Using the ammonia diffused from the standard solution as a reference, the ammonia diffused from the blood sample at any one time was relatively constant as shown in Table I and Fig. 4. This is interpreted to mean that the rate of diffusion of ammonia from blood is essentially the same as from water in this system, and that there is negligible hydrolysis of the amides in blood to produce ammonia. If hydrolysis had occurred the blood ammonia would have increased with time. This was the case, as will be shown below, when stronger alkali was used to liberate ammonia. A time-diffusion curve of blood plus added ammonia was similar to that of blood alone as shown in Fig. 3 and Table I. The blood ammonia observed in this case for the varying times of diffusion was also relatively constant as found with blood alone. The ammonia recovered at the varying times of diffusion was also constant. These data indicate that the rate of diffusion of ammonia from blood or blood plus ammonia is the same as that from the aqueous standard. Hydrolysis of amides to yield ammonia appears to be negligible because the blood ammonia did not rise significantly with exposure to the buffered alkali.

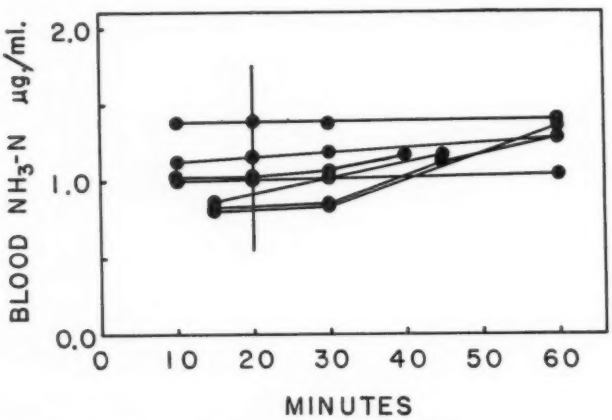

Fig. 4.—The effect of $K_2CO_3 \cdot 1\frac{1}{2}\ H_2O$ plus $KHCO_3$ on blood ammonia content with increasing time of diffusion. The points shown were calculated from comparisons with ammonia standards run simultaneously for the same interval. The line at 20 minutes represents the period of time selected for diffusion in the proposed method.

In order to establish a suitable time for diffusion, several samples of blood from patients with various diseases were diffused for 10 to 60 minutes. The blood ammonia was estimated from concurrent standards set up for the same diffusion times. The data so derived are shown in Fig. 4. The blood ammonia in these samples was constant between 10 and 30 minutes for all samples and constant for many samples for as long as 60 minutes. The time of the diffusion was arbitrarily set at 20 minutes to avoid the increase in blood ammonia occurring with prolonged exposure to alkali.

Recovery[1] of added ammonia was calculated using the amount of ammonia estimated to be present after addition as 100 per cent. The quantity found ranged from 93 to 107 per cent (Table I). In 100 consecutive analyses, duplicates differed by an average of 2.5 per cent from the mean.

TABLE I. EFFECT OF $K_2CO_3 \cdot 1\frac{1}{2}\ H_2O$ AS AN ALKALINIZING AGENT ON RATE OF DIFFUSION OF AMMONIA FROM BLOOD AND RECOVERY OF ADDED AMMONIA. CLINICAL DIAGNOSIS: SUBJECT 7, NORMAL; SUBJECT 8, CIRRHOSIS; SUBJECT 9, NORMAL; AND SUBJECT 10, BANK BLOOD, 60 DAYS OLD

			AMMONIA ADDED			
SUBJECT NUMBER	TIME DIFFUSED MINUTES	BLOOD NH_3-N MCG. PER ML.	FOUND IN BLOOD NH_3-N MCG. PER ML.	ADDED NH_3-N MCG. PER ML.	RECOVERED NH_3-N MCG. PER ML.	RECOVERY OF ESTIMATED TOTAL NH_3-N* PER CENT
7	20	1.00	3.78	2.50	2.78	105
	30	1.06	3.68		2.62	102
	60	1.05	3.35		2.30	97
8	10	1.02	3.95	2.50	2.93	107
	20	1.03	3.78		2.75	104
	30	1.16	3.58		2.42	99
9	15	0.89	3.48	2.50	2.59	101
	30	1.03	3.31		2.28	96
	45	1.17	3.30		2.13	93
10	20	16.0	23.8	10.0	7.8	94
	30	15.3	23.9		8.6	96
	60	15.1	22.7		7.6	93

*X = fraction of NH_3-N recovered from the total amount estimated to be present.
A = mcg. NH_3-N added.
B = mcg. NH_3-N found in blood.
C = mcg. NH_3-N found in the blood to which A was added.

$\frac{C}{X}$ = true NH_3-N in blood to which A was added.

$\frac{B}{X}$ = true NH_3-N in original blood.

$$\frac{\frac{C}{X}}{\frac{B}{X} + A} = X$$

$$X = \frac{-B + \sqrt{B^2 + 4\ A\ C}}{2\ A}$$

Study of the Adverse Effects of K_2CO_3 Used as an Alkalinizing Agent.—Conway[1, 7] used K_2CO_3 to alkalinize blood for liberation of ammonia. He allowed 10 minutes for diffusion and measured the collected ammonia titrimetrically. Because ammonia diffused more slowly from blood than from water, he applied a correction factor of 1.15 to the measured ammonia. He then made a negative correction for "the special deaminating action of the alkali" which at 25° C. was 0.11 mcg. per ml. blood. Under these circumstances he found no ammonia in freshly drawn blood. When the blood was exposed to air for 5 minutes so that CO_2 was lost he found a mean value of 0.47 mcg. of NH_3-N per ml. for human blood. He called this easily split-off ammonia "alpha ammonia" which forms after "shedding" of CO_2. A recent report by Schwartz and associates[12] used the Conway method but allowed 60 minutes for diffusion and applied no corrections. They reported a range of 1.0 to 3.0 mcg. NH_3-N per ml. of blood. McDermott and Adams[13] reported data from a modified Conway procedure. These authors did not indicate the use of the correction factors of Conway but did diffuse for 10 minutes. Their values ranged from 0.499 to 0.788 for normal human blood with an average of 0.608.

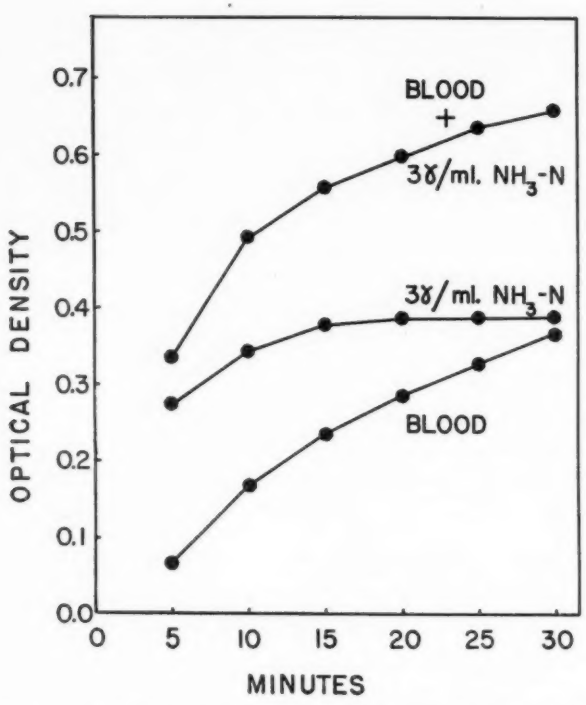

Fig. 5.—The effect of unbuffered K_2CO_3 as an alkalinizing agent to facilitate diffusion of ammonia from freshly drawn blood; the same blood plus 3.0 mcg. NH_3–N per ml. and water containing 3.0 mcg. NH_3–N per ml. The abscissa represents the time of diffusion which also indicates the time of contact between the alkali and the blood.

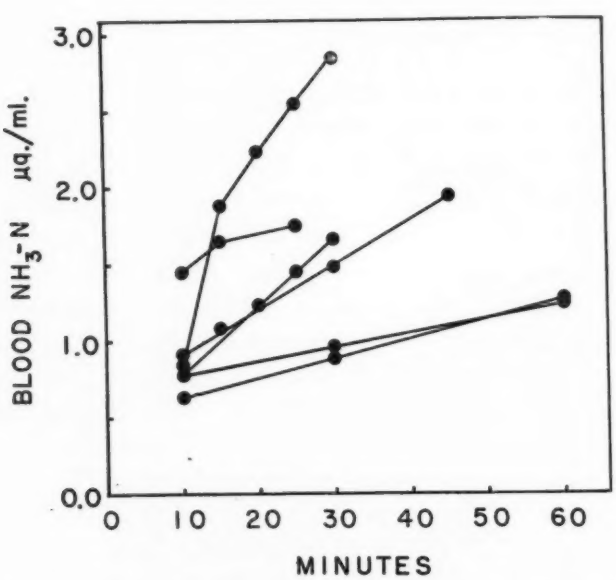

Fig. 6.—The effect of K_2CO_3 on blood ammonia with increasing time of diffusion. The points shown were calculated from comparisons with ammonia standards run simultaneously for the same interval.

These results are cited to indicate the irregularity of values obtained with the Conway procedure when applied differently by 3 laboratories. We believe that this is in part a result of the action of K_2CO_3 which causes hydrolysis of labile amides in blood. In order to study this effect in the proposed method, 2 Gm. of K_2CO_3 were used for alkalinizing blood and the $KHCO_3$ was omitted. As shown in Fig. 5 (compare with Fig. 3), the ammonia continued to increase with time as the diffusion and contact with unbuffered alkali continued. The diffusion of ammonia from the standard, however, was complete at 15 to 20 minutes. Calculation of ammonia from the concurrent standards showed a rise of blood ammonia with duration of diffusion as shown in Fig. 6 and Table II. This increase (up to 300 per cent), found in the presence of

TABLE II. THE EFFECT OF $K_2CO_3 \cdot 1\frac{1}{2}\ H_2O$ AS AN ALKALINIZING AGENT ON RATE OF DIFFUSION AND FORMATION OF AMMONIA FROM BLOOD; AND RECOVERY OF ADDED AMMONIA. CLINICAL DIAGNOSIS: SUBJECTS 1, 2, 3, AND 5 ARE HEALTHY ADULTS; SUBJECT 4 SAME SAMPLE AS SUBJECT 3, BUT ANALYSIS PERFORMED 4 HOURS LATER

			AMMONIA ADDED			
SUBJECT NUMBER	TIME DIFFUSED MINUTES	BLOOD NH_3-N MCG. PER ML.	FOUND IN BLOOD NH_3-N MCG. PER ML.	ADDED NH_3-N MCG. PER ML.	RECOVERED NH_3-N MCG. PER ML.	RECOVERY OF ESTIMATED TOTAL NH_3-N[1] PER CENT
1	10	0.46	1.85	1.50	1.39	97
	15	0.54	1.99		1.45	99
	30	0.74	2.23		1.49	100
2	5	0.34	1.59	1.50	1.24	92
	10	0.48	1.77		1.29	94
	15	0.52	1.91		1.39	97
	30	0.54	2.10		1.56	102
3	5	0.38	1.84	1.50	1.46	99
	10	0.72	2.16		1.44	98
	15	0.95	2.22		1.27	94
	20	1.12	2.32		1.20	92
	25	1.21	2.46		1.19	94
	30	1.43	2.55		1.12	91
4	5	1.50	2.22	1.50	0.72	82
	10	1.79	2.71		0.92	87
	15	1.97	2.92		0.95	89
	20	2.08	3.06		0.98	89
	25	2.22	3.29		1.07	91
	30	2.30	3.41		1.11	92
5	5	0.44	1.53	2.50	1.09	70
	10	0.72	1.77		1.05	71
	15	0.99	1.93		0.99	70
	20	1.16	2.08		0.92	71

strong alkali (K_2CO_3 alone), was in sharp contrast to the constant amount of ammonia found when $KHCO_3$ was added to reduce alkalinity (compare Fig. 6 with Fig. 4 and Table II with Table I). Recovery of ammonia added to blood amounted to 70 to 102 per cent. Occasional low, unexplained recoveries were encountered with K_2CO_3 (Table II) but not with the buffered salts. The rise of blood ammonia with time is thought to be the result of hydrolysis of amides. Glutamine alone is sufficiently high in concentration to yield 5 times the normal blood ammonia when it is hydrolyzed.

Study of Bank Blood.—It was observed that sterile blood from the blood bank, properly refrigerated, developed ammonia on standing, presumably because of enzymatic hydrolysis of amides, although other reactions may have contributed. Such a blood which had been stored for 60 days was treated with 2 Gm. of $K_2CO_3 \cdot 1\frac{1}{2}\ H_2O$ and a time-diffusion curve was made (Fig. 7). The large amount of ammonia was diluted with Nessler reagent for colorimetry. In this experiment, maximum diffusion from the standard occurred in 15 minutes. The 15-minute blood ammonia value was 16.7 mcg. as compared with the 30-minute value of 17.7 mcg. The curve with added ammonia was similar (Fig. 7).

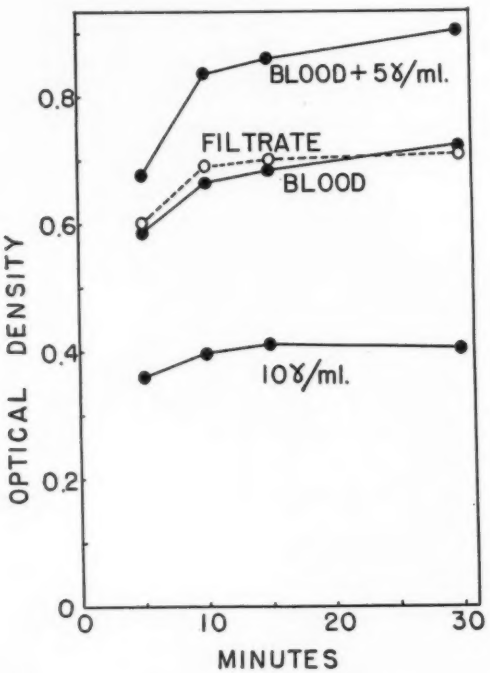

Fig. 7.—The effect of K_2CO_3 as an alkalinizing agent to facilitate diffusion of ammonia from 60-day-old bank blood; the same blood plus 5.0 mcg. NH_3–N per ml.; and water containing 5.0 mcg. NH_3–N per ml. The dotted line represents the protein-free filtrate. The abscissa represents the time of diffusion.

A protein-free filtrate (made with tungstic acid) of this blood showed a time-diffusion curve which differed only slightly from the aqueous standard. This difference was interpreted to mean that bank blood had lost most of the hydrolyzable substances in storage but still contained a small amount which could be split by carbonate. It should be noted that when blood was alkalinized with K_2CO_3, the production of ammonia as diffusion progressed was considerably lower in bank blood than in fresh blood (compare Figs. 5 and 7).

A comparison of the diffusion curve (Fig. 7) of the blood-carbonate mixture with the protein-free filtrate-carbonate mixture suggests that the blood

proteins retard ammonia diffusion. This observation was made several times and can be seen in Fig. 5. Conway[1, 7] observed this retarding effect of blood proteins and applied a correction factor of 1.15 to his analytical results.

Other methods which use K_2CO_3 to alkalinize blood may produce artifacts resulting from hydrolysis or retardation of the rate of diffusion of ammonia. The difference between the blood ammonia content of normal persons reported by Conway[1, 7] (0.0 to 0.5 mcg. per ml.) and that of Schwartz[12] (1.0 to 3.0 mcg. per ml.), using the same method, may be due to the longer exposure to K_2CO_3 in the analysis as used by the latter group.

Is Ammonia in Blood an Artifact?—Conway reported that fresh blood, when handled anaerobically to prevent loss of CO_2, contained no free ammonia. On exposure to air so that CO_2 was lost, ammonia appeared and reached values up to approximately 0.5 mcg. per ml. as shown in Fig. 8 with reconstructed curves from his published data. To test this observation, 2 ml. of fresh venous blood drawn anaerobically were injected into the prepared diffusion vessel for ammonia analysis within 20 seconds from the time of venipuncture. Blood

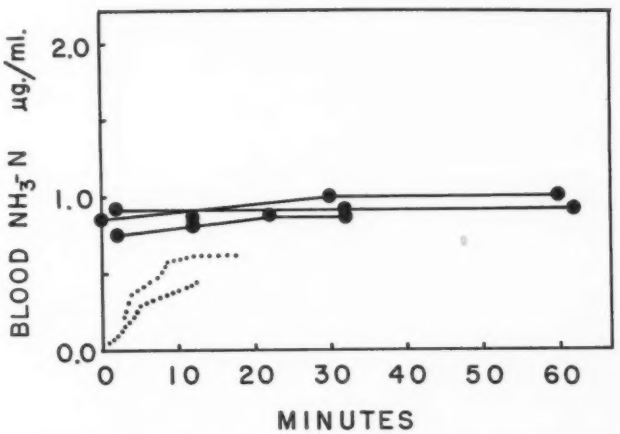

Fig. 8.—Ammonia content in freshly drawn blood exposed to air as measured by the prescribed method. The dotted lines are two reconstructed, typical curves from the data of Conway[1, 7] indicating ammonia formation in exposed blood as measured by his method. The abscissa represents the time that blood was exposed to air before analysis.

from the same source was then put into an open beaker at 23° C. and analyzed periodically for 300 minutes. Ammonia rose at a constant rate of 0.003 mcg. per ml. of blood per minute. The data from similar experiments are plotted in Fig. 8. Essentially the same amount of ammonia was obtained at zero time as at 60 minutes, indicating very little ammonia formation after exposure to air. Others[2-6, 12, 13] mention the presence of ammonia in freshly drawn blood. Kirk,[2] in his monograph, described ammonia formation in normal bloods on standing to be 0.007 mcg. NH_3-N per ml. of blood per minute, whereas in 2 patients with cirrhosis of the liver it was twice this value. Conway mentioned that ammonia formation on standing was 0.01 mcg. per ml. per minute. It would appear that the ammonia measured in blood in this experiment shown in Fig. 8 at zero time is *free* or *true* blood ammonia and not easily split-off ammonia. The slight and

almost negligible formation of ammonia on standing is presumed to be the result of enzymatic hydrolysis. The extra ammonia that forms on contact with carbonate on the other hand is the result of alkaline hydrolysis. The results of Conway (Fig. 8) which indicate the absence of ammonia in fresh blood differ from the data presented in this report (Fig. 8). This difference is unexplained.

The Measurement of Erythrocyte and Plasma Ammonia.—Since ammonia formation in standing whole blood appears to be slight and a direct function of time this was assumed to be true of erythrocyte and plasma. Heparinized blood was analyzed for its ammonia content, a portion was separated into plasma and red cells by centrifuging at 3,000 r.p.m. for 20 minutes, and these were analyzed for their ammonia content. Analyses were repeated 20 minutes later to estimate ammonia formation in this period. The ammonia formed per minute in each fraction was calculated and the content of blood, plasma, and red cells was extrapolated to the time of venipuncture from the rate of hydrolysis as determined for ecah sample. Table III shows the data so obtained. The ammonia values in the plasma and erythrocytes were corrected for hematocrit, added together, and found to equal the directly measured ammonia content of whole blood. It appears that erythrocytes contain 2 to 3 times as much ammonia as plasma. Seegmiller and associates[14] report the mean plasma value to be 1.3 meg. per ml. and the range to be 0.5 to 2.2, values which are higher than reported in Table III.

TABLE III. AMMONIA CONTENT OF WHOLE BLOOD, PLASMA, AND ERYTHROCYTES BY DIRECT MEASUREMENT ON THESE BLOOD FRACTIONS

SUBJECT	VOL. PACKED CELLS PER CENT	AMMONIA CONTENT			
		WHOLE BLOOD MCG. PER ML.	PLASMA MCG. PER ML.	ERYTHROCYTES MCG. PER ML.	ERYTHROCYTES PLASMA RATIO
K. J.	43.5	0.98	0.49	1.41	2.9
D. S.	47.5	0.94	0.48	1.47	3.1
G. McC.	45	0.78	0.44	1.20	2.7
C. O.	43	0.84	0.51	1.28	2.5

TABLE IV. AMMONIA CONTENT OF BLOOD, PLASMA, AND ERYTHROCYTES BY MEASUREMENT OF BLOOD AND PLASMA AMMONIA AND CALCULATION OF ERYTHROCYTE AMMONIA. THESE 29 SUBJECTS WERE HOSPITALIZED PATIENTS FREE OF LIVER DISEASE

	WHOLE BLOOD MCG. PER ML.	PLASMA MCG. PER ML.	ERYTHROCYTES MCG. PER ML.	ERYTHROCYTES PLASMA RATIO
Mean	1.37	0.78	2.20	2.81
Standard Deviation	0.26	0.17	0.11	0.88
Range	0.75-1.96	0.56-1.22	0.96-3.31	1.00-4.71

Table IV contains some values for whole blood, erythrocytes, and plasma ammonia observed in 29 hospitalized patients free of liver disease by the proposed analytical method; that is, measurement of ammonia in blood and plasma and calculation of ammonia in erythrocytes.

DISCUSSION OF RESULTS

The method described appears to have certain advantages for the determination of blood ammonia as discussed above. Although the NH_3-N content of 2 ml. of normal blood is approximately 2 mcg., it is relatively simple under the conditions described to isolate most of it by diffusion and measure it colorimetrically. The method is applicable to other delicate systems besides blood. The mixture of $K_2CO_3 \cdot 1\frac{1}{2}\ H_2O$-$KHCO_3$ saturates the blood and causes the pH to rise to 11.0 to 11.5. Under these conditions, the rates of diffusion of ammonia from blood and from aqueous standard were found to be the same. Also, the rate of hydrolysis in this buffered alkaline mixture during the first 20 minutes of diffusion is so slight as to be negligible. No corrections for diffusion or hydrolysis need be applied. The slight formation of ammonia in blood on standing at room temperature appears to be due to enzymatic hydrolysis of one or more blood constituents. On long standing, even in the sterile state, the free ammonia can rise 10 or more times its initial value. On the other hand, blood mixed with K_2CO_3 alone yields ammonia rapidly and excessively because of alkaline hydrolysis. The method for meauring ammonia in urine,[10] from which this method was derived, cannot be used for measuring blood or plasma ammonia because of this factor of hydrolysis by K_2CO_3. The ammonia values so obtained[14] for plasma are up to 400 per cent higher than those cited in Table III.

It is believed that the ammonia measured under these circumstances is real and not artifactitious. The observed normal ammonia values appear to be higher than some reported[1-7, 13] and lower than others.[12, 14] The lower values reported by some might be explained by the inhibiting effects of proteins on the rate of diffusion of ammonia. The higher values reported may be due to enzymatic hydrolysis of blood amides before analysis or alkaline hydrolysis during analysis to produce more ammonia than was originally present.

The observation that erythrocytes concentrate ammonia is unexplained. Fenn and co-workers[15] have shown that muscle incubated in ammonium chloride solutions picks up ammonia. After 4 to 5 days, the muscle ammonia rose to 1.5 times that in the incubating solution. Similar observations were made in liver, smooth muscle, and skin.

SUMMARY

1. A new method for the measurement of ammonia in blood, erythrocytes, plasma, or other labile biologic systems has been described.

2. Conditions which produce falsely high or low values encountered in other methods are avoided.

3. The ammonia found in freshly drawn human venous blood is believed to be preformed true ammonia, present as ammonium ions and not an artifact.

4. The ammonia content of blood, erythrocytes, and plasma in 29 patients free of liver disease is reported.

5. Ammonia in erythrocytes is 2.8 times that found in plasma.

The authors are deeply indebted to Miss Jean Marino for some experimental work, to Mrs. Harriet Seligson, Dr. John Reinhold, and Dr. David Drabkin for their help in editing.

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Erratum

In the article entitled "The Interrelations of Blood Calcium and Citric Acid" by John J. Canary, M.D., and Laurence H. Kyle, M.D., pages 590-596 of the April issue of the JOURNAL, the next to last sentence on page 593 should read as follows: "Infusions of citric acid were reported to result in an elevation of calcium by Chang and Freeman,[28] while Gomori and Guylas[27] found no change."

DIFCO

why

BACTO-THROMBOPLASTIN

is the most widely used agent for prothrombin determinations

17 years' use *has demonstrated its*

1) *accuracy and reliability*

2) *high sensitivity and potency*

3) *stability and dependability*

4) *simplicity of preparation*

5) *economy*

Each box of Bacto-Thromboplastin contains 6 ampules, sufficient for 210 Quick determinations or 420 Link-Shapiro determinations.

BACTO-THROMBOPLASTIN is a stabilized desiccated rabbit brain substance of high thromboplastin potency. It is used in all prothrombin time procedures with either whole or diluted plasma. Bacto-Thromboplastin is stable in either dry form or frozen extract.

Descriptive literature gladly sent upon request.

DIFCO LABORATORIES

DETROIT 1, MICHIGAN

THIS IS

NEEDLETAINER

by Sterilon

Available for most needle sizes. Gauge and length are stamped on cap or blank caps are available.

STERILON'S REUSEABLE NYLON CONTAINER THAT KEEPS NEEDLES STERILE, DAMAGE-FREE AND READY FOR INSTANT USE . . . INDEFINITELY

Edge Under Needletainer Cap is Sterile, Permitting Pouring of Needle on to Sterile Field. Or, Syringe May be Inserted for Attaching Sterile Needle Aseptically and Withdrawing Ready for Use.

TAKE OFF CAP...

INSERT SYRINGE...

TWIST AND REMOVE

Sterilon Needletainers bring new efficiency to needle sterilization, storage, handling and use.

To use, simply put cleaned needle into Needletainer, wet or dry. Snap on cap and place in autoclave for a *30 minute cycle.* Needle will then be sterile and will remain so until cap is removed.

Unbreakable Needletainers protect needle from damage. Compact size permits easy storage or carrying. Can be used and re-used for keeping needles sterile as long as cap fits tightly. (Pat. Pending)

No. 95467 NEEDLETAINER, complete with caps. per dozen-$.60

In 5 gross lots per dozen-$.54

(Minimum Order, 4 Dozen)

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STANDARD SCIENTIFIC

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Sigmamycin*

OLEANDOMYCIN TETRACYCLINE

provides added certainty in antibiotic therapy particularly for that 90% of the patient population treated in home or office...

Multi-spectrum synergistically strengthened SIGMAMYCIN provides the antimicrobial spectrum of tetracycline extended and potentiated with oleandomycin to include even those strains of staphylococci and certain other pathogens resistant to other antibiotics.

Supplied: SIGMAMYCIN CAPSULES – 250 mg. (oleandomycin 83 mg., tetracycline 167 mg.), bottles of 16 and 100; 100 mg. (oleandomycin 33 mg., tetracycline 67 mg.), bottles of 25 and 100. SIGMAMYCIN FOR ORAL SUSPENSION – 1.5 Gm., 125 mg. per 5 cc. teaspoonful (oleandomycin 42 mg., tetracycline 83 mg.), mint flavored, bottles of 2 oz.

*Trademark

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Division, Chas. Pfizer & Co., Inc.
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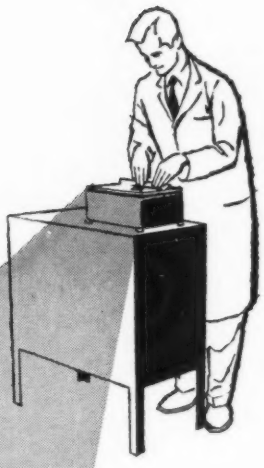

aloe

NEW **MICRO-STROPPER**

ELECTRIC MICROTOME KNIFE STROPPER*

STROPS KNIFE TO KEEN, UNIFORM CUTTING EDGE

Much faster and more accurate than old hand-stropping methods. Merely run blade over special compounded wheel four or five times each side. Knife holder and support plate adjustable for differences of bevel on various knives. For further data write for Technical Bulletin 23-122.

60070. Grey finished housing, 13½ inches wide, 11 inches deep, 9 inches high. For 115 volts A. C. Complete with charged stropping wheel and extra supply of stropping abrasive for recharging. Each $275.00

*Patent applied for

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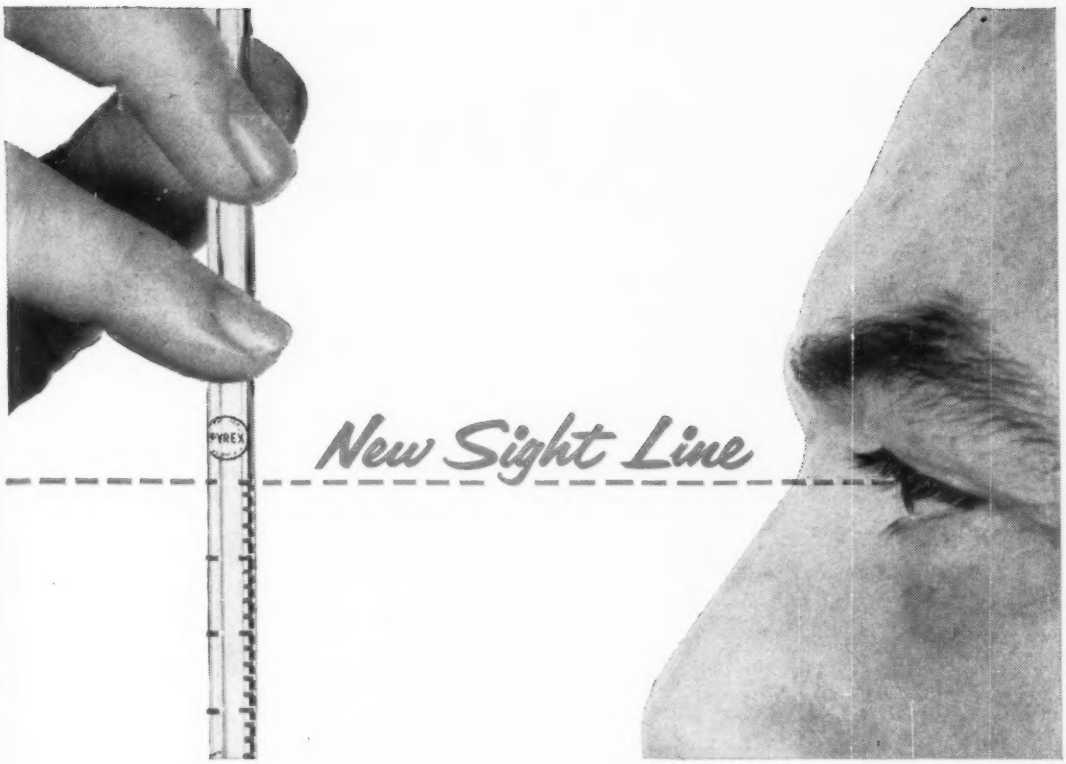

Save 15% with new PYREX brand Accu-red pipettes

... get faster sight line readings, too

You can save up to $5.18 every time you order a package of 18 *Accu-red* measuring (Mohr type) or serological pipettes.

Oddly enough, it's the fact that these pipettes are so accurate that makes them cost you less. We've found a way of making fine bore tubing with such uniform inner diameters that we can calibrate and mark these pipettes much faster.

Accu-red pipettes cost us less to make, so they cost you less to buy.

You get sight lines, too. You'll notice on the back of *Accu-red* pipettes short lines which level perfectly with the major graduations. Use them—like gun-sights—to get faster, more accurate readings.

Both graduations and sight lines are in permanent LIFETIME RED. Actually part of the glass itself, LIFETIME RED markings stay sharply legible for the life of the pipette.

Plus the PYREX trademark. Because *Accu-red* pipettes are made of chemically resistant PYREX brand glass No. 7740, they will not alter antigens, or biological or chemical solutions. Nor are they affected by most cleaning solutions. And, PYREX brand pipettes withstand sterilizations, both wet and dry.

Like all pipettes bearing the PYREX trademark, *Accu-red* pipettes have extra strength *built-in*—double-bevelled tips, ground satin-smooth, reduce snag-induced tip breakage; uniform walls, unmarred by etched graduations, produce a stronger pipette from mouthpiece to tip.

Sizes and graduated intervals. 7085 serological pipettes, 1 ml capacity graduated in 1/10 ml, 1 x 1/100, 2 x 1/10, 5 x 1/10, and 10 x 1/10. 7065 Mohr type, same as 7085 plus 25 ml in 1/10 ml.

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new!!...a colorimetric tablet test for proteinuria **Albutest®**

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entirely new concept ALBUTEST detects protein by a blue color change on the yellow tablet surface instead of by the usual estimation of degrees of turbidity. ALBUTEST permits use of a color guide which—for the first time—provides points of reference for securing standardized results.

unaffected by urine turbidity Filtration or centrifuging not required.

sensitive and specific ALBUTEST detects clinically significant albuminuria—does not react with normal urines or with any urine constituent other than protein.

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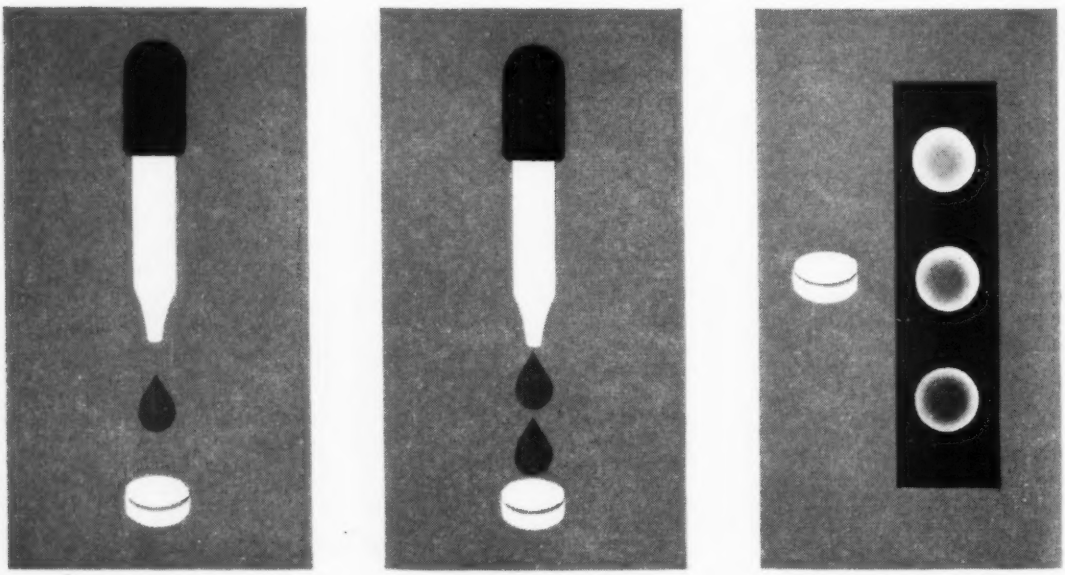
economical and convenient No test tubes, reagent bottles, burners or other equipment needed. Saves personnels' time usually lost in ordinary testing and in cleaning of equipment. Simple—only a few seconds and an ALBUTEST Reagent Tablet needed.

1 One drop urine on ALBUTEST Tablet

2 Two drops water

3 Compare with color guide



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in balances...

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For example, what other lab instruments can give you 6 or 7 significant figures like an analytical balance... and gravimetric methods are ubiquitous.

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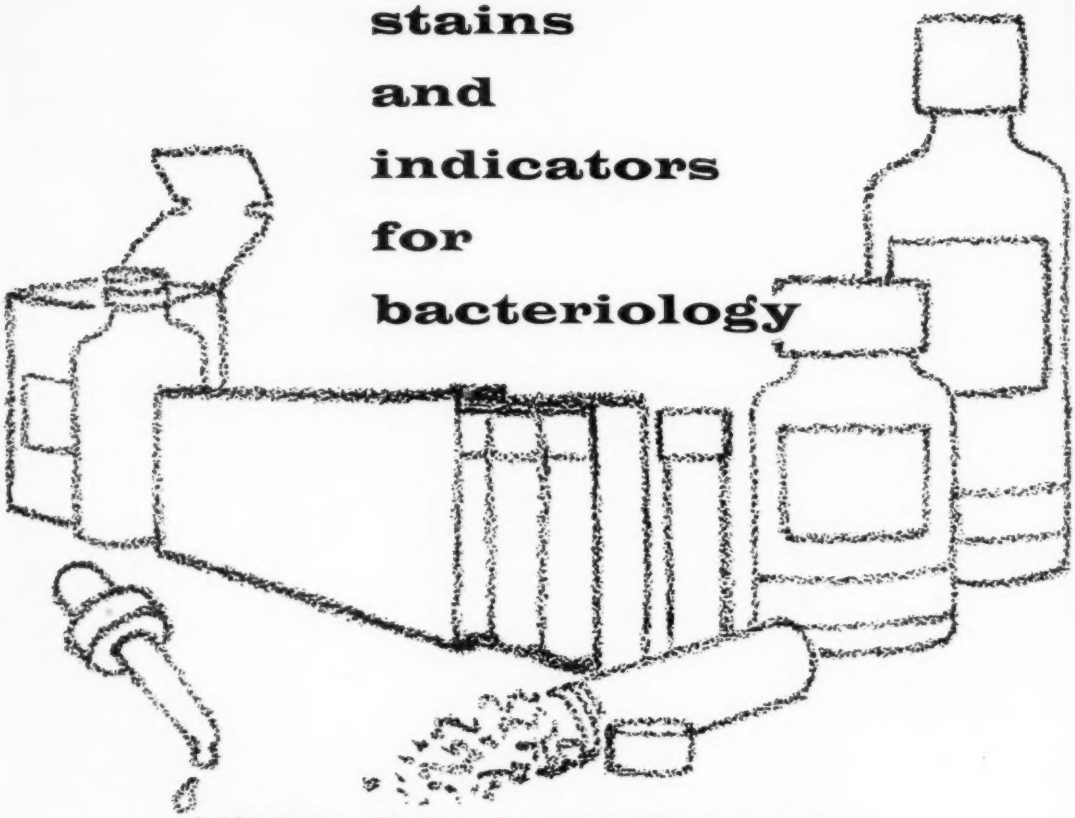
HARLECO

stains and indicators for bacteriology



Harleco offerings for bacteriological studies embrace a wide selection of stains that have been approved by the Biological Stain Commission for the staining of bacteria; in certain cases for suitable bacteriostatic action. Another group of dye products used broadly in bacteriology are the sulfonphthalein dyes as indicators in the adjustment of culture media. This latter group finds favor in the Harleco "WS" (water-soluble) form since this form provides the ultimate in convenient use.

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Lightens the laboratory work load . . . this team of Coleman Instruments not only lightens the present work load with faster, more dependable analyses . . . but prepares the laboratory for the even greater demands on hospital and laboratory facilities that come with expanding medical services.

Wider professional scope for the technologist . . . this reliable *proven* instrumentation brings greater confidence and speed . . . *plus* access to the many new and improved analytical technics developed by prominent authorities *on and for Coleman Instruments.**

The Coleman Junior Spectrophotometer stands throughout the world as the unquestioned choice of analytical instruments for the clinical laboratory. Fifteen years of dependable service—uncounted volumes of analytical methods stand behind this preference.

The Coleman Flame Photometer is the only instrument designed specifically for the direct measurement of Sodium, Potassium and Calcium in the biological samples encountered in the clinical laboratory.** Together, the Coleman Junior Spectrophotometer and the Coleman Flame Photometer provide the finest and most dependable of all analytical tools for clinical analysis—and at the lowest possible cost.

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* ☐ A Colorimetric Method for SGO Transaminase on the Coleman Junior Spectrophotometer—Bulletin T-143.

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Harris Hematoxylin (modified)

Papanicolaou Stains prepared according to the original formulae for the cytological diagnosis of cancer by means of the smear technic.

These stains conform to Paragon's rigid standard of excellence in every way at a modest cost that renders preparation by the laboratory technician unnecessary.

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Each lot of stain is tested against smears in our laboratories for correct differential staining, color balance and transparency.

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PAPANICOLAOU STAIN—PARAGON EA-36
For general staining of vaginal and cervical smears and in endocrine studies.

PAPANICOLAOU STAIN—PARAGON EA-65
For staining smears containing much mucus as sputum, gastric and pleural fluids, etc. Similar to EA-36 but yielding better differentiation in the presence of mucus.

PAPANICOLAOU STAIN—PARAGON OG-6
The Orange G stain for use with EA-36 and EA-65 in the Papanicolaou technic.

HARRIS HEMATOXYLIN—PARAGON (modified)
For Papanicolaou Staining
A modified ready to use Harris Hematoxylin Stain specially formulated for Papanicolaou staining. It yields a sharp blue nuclear stain with no staining of the cytoplasm.

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PAPANICOLAOU STAINS—PARAGON are packed in two convenient sizes only, a 250 c.c. and a 500 c.c. bottle.

Name	Catalog No.	500 c.c. Bottle	250 c.c. Bottle
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PAPANICOLAOU STAIN—PARAGON EA-36	PS1282	3.85	
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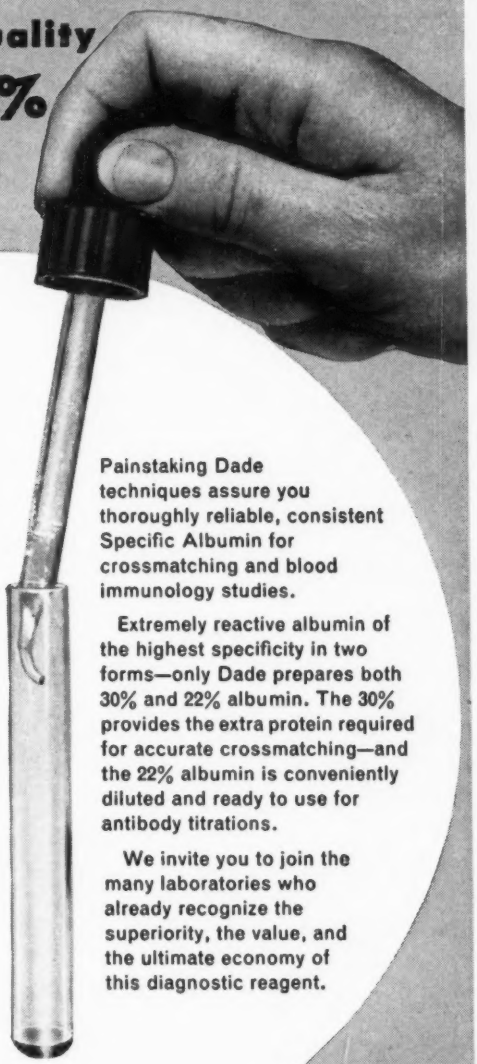
For Crossmatching and Antibody Studies.



Painstaking Dade techniques assure you thoroughly reliable, consistent Specific Albumin for crossmatching and blood immunology studies.

Extremely reactive albumin of the highest specificity in two forms—only Dade prepares both 30% and 22% albumin. The 30% provides the extra protein required for accurate crossmatching—and the 22% albumin is conveniently diluted and ready to use for antibody titrations.

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R

HYLAND TECHNI-GRAM

blood grouping literature reflects growing interest in

LECTINS*

(group specific hemagglutinins of botanical origin)

Particular attention has focused on the high degree of blood group specificity shown by extracts (lectins) of certain seeds. The seed of *Dolichos biflorus*, for example, contains a lectin of unusual avidity and specificity for blood specimens of subgroups A_1 and A_1B. Other lectins of other specificities have also been reported.

*Boyd [1, 2] proposed the name "lectin" (from the Latin *legere*, meaning to choose or pick out) for these "antibody-like" substances which react specifically with certain antigens. This name was selected to call attention to their specificity, while differentiating them from true antibodies.

1. Boyd, W.C.: Fundamentals of Immunology, 3rd Ed. Interscience, New York-London, 1956.
2. Boyd, W. C., and Shapleigh, E.: J. Immunol. **73**: 226-231 (1954).

Announcing the first commercial preparation of

ANTI-A_1 LECTIN

(Purified)

This new Hyland product is a water-clear, stable extract purified from the seed of *Dolichos biflorus* and specific for subgroups A_1 and A_1B.

Hyland Anti-A_1 Lectin (Purified) produces rapid and clear-cut agglutination of A_1 and A_1B specimens by either slide or test tube methods; A_2 and A_2B specimens form a smooth suspension.

Once you try this new, fast-acting, specific product you will use it routinely. Available in 2 cc vials.

Hyland

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. . . the eighteen pound, transistorized model 300 VISETTE

For the clinical accuracy your heart practice demands . . . *and a degree of portability never before approached in the field of 'cardiography* . . . this new Sanborn instrument offers a truly remarkable answer.

In the VISETTE you will find outstanding Sanborn quality and performance, achieved through the latest electronic techniques and the most modern principles of instrumentation. Tiny transistors largely replace bulky vacuum tubes . . . entire circuits are contained in plug-in printed wiring panels no larger than a playing card . . . 'cardiograms are clearly traced on chart paper in a new, convenient width. Innovations such as these have also made possible economies in production, reflected in the comparably lower price of the new 300 VISETTE.

Every design feature, every component in this modern instrument, serves a single purpose: clinically *accurate* 'cardiograms with the greatest possible *convenience*. The "Sanborn man" in or near your city can provide complete details, and a demonstration in your office if you wish. And of course you may try a VISETTE (as you can other Sanborn instruments)—*before* buying, without cost or obligation.

To those who already own the famous Model 51 Viso-Cardiette, the new VISETTE can be an invaluable "companion" ECG — especially suited to use outside the office, or in hospital wards. Or, for those who prefer a larger instrument, using conventional 6 cm. width recording paper, the "51" is still available at $785 delivered.

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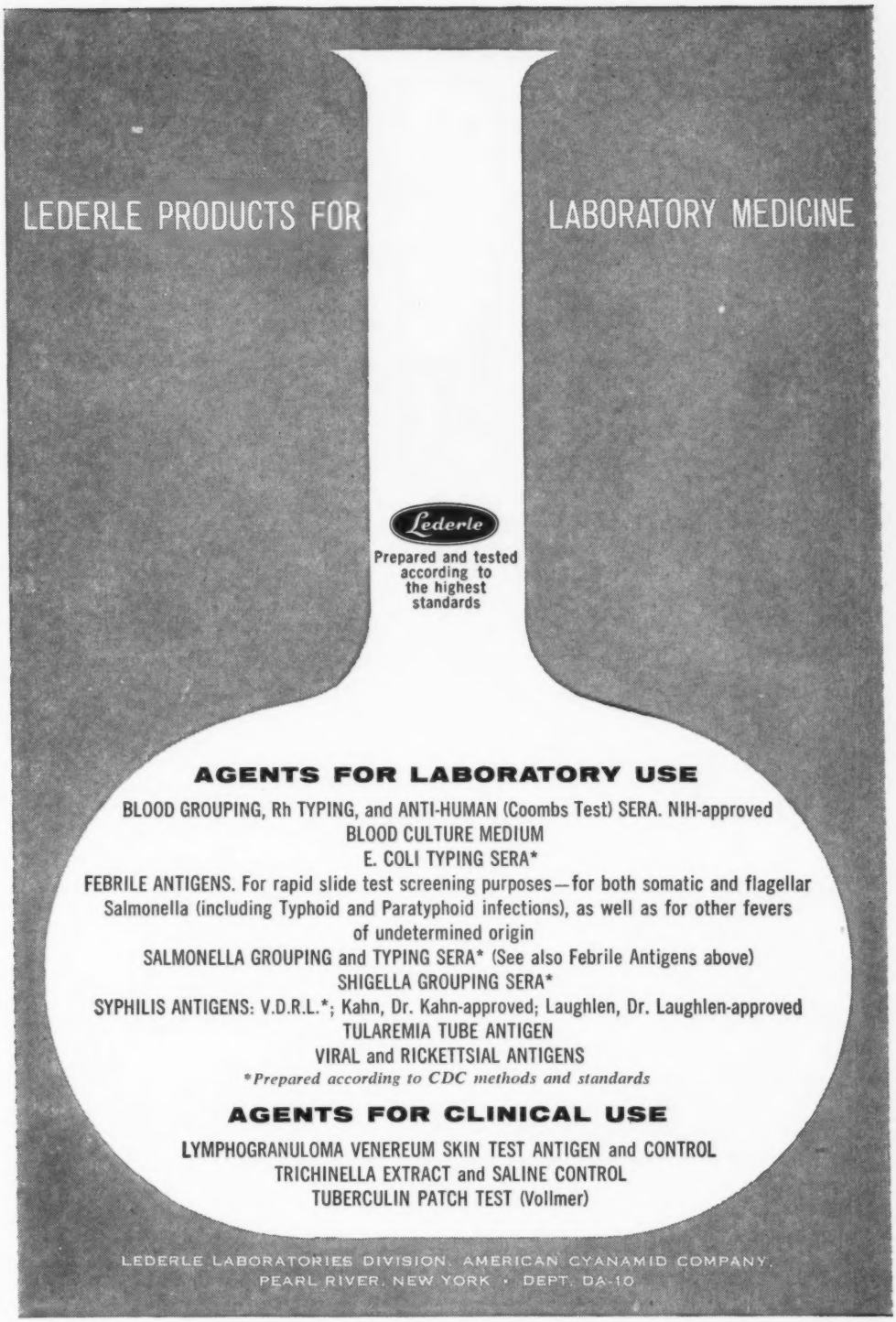
WALTHAM 54, MASS.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Prepared and tested according to the highest standards
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BLOOD GROUPING, Rh TYPING, and ANTI-HUMAN (Coombs Test) SERA. NIH-approved
BLOOD CULTURE MEDIUM
E. COLI TYPING SERA*
FEBRILE ANTIGENS. For rapid slide test screening purposes—for both somatic and flagellar Salmonella (including Typhoid and Paratyphoid infections), as well as for other fevers of undetermined origin
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TULAREMIA TUBE ANTIGEN
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*Prepared according to CDC methods and standards
AGENTS FOR CLINICAL USE
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Heat and moisture-sensitive supplies too delicate for conventional processing may now be Steroxcide sterilized in the hospital countless times without injury. Substantial savings in sterile supply costs will result.

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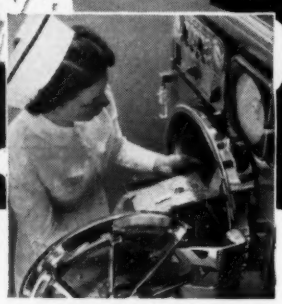
Now, there is nothing that cannot be sterilized 100%.

Newly developed water ejection unit speeds cycle, prepares spore-bearing organisms for killing action of gas.



Pre-packaged materials may be processed and distributed in their own containers for indefinite sterile storage.

For additional information write for Catalog Section 4 (T).

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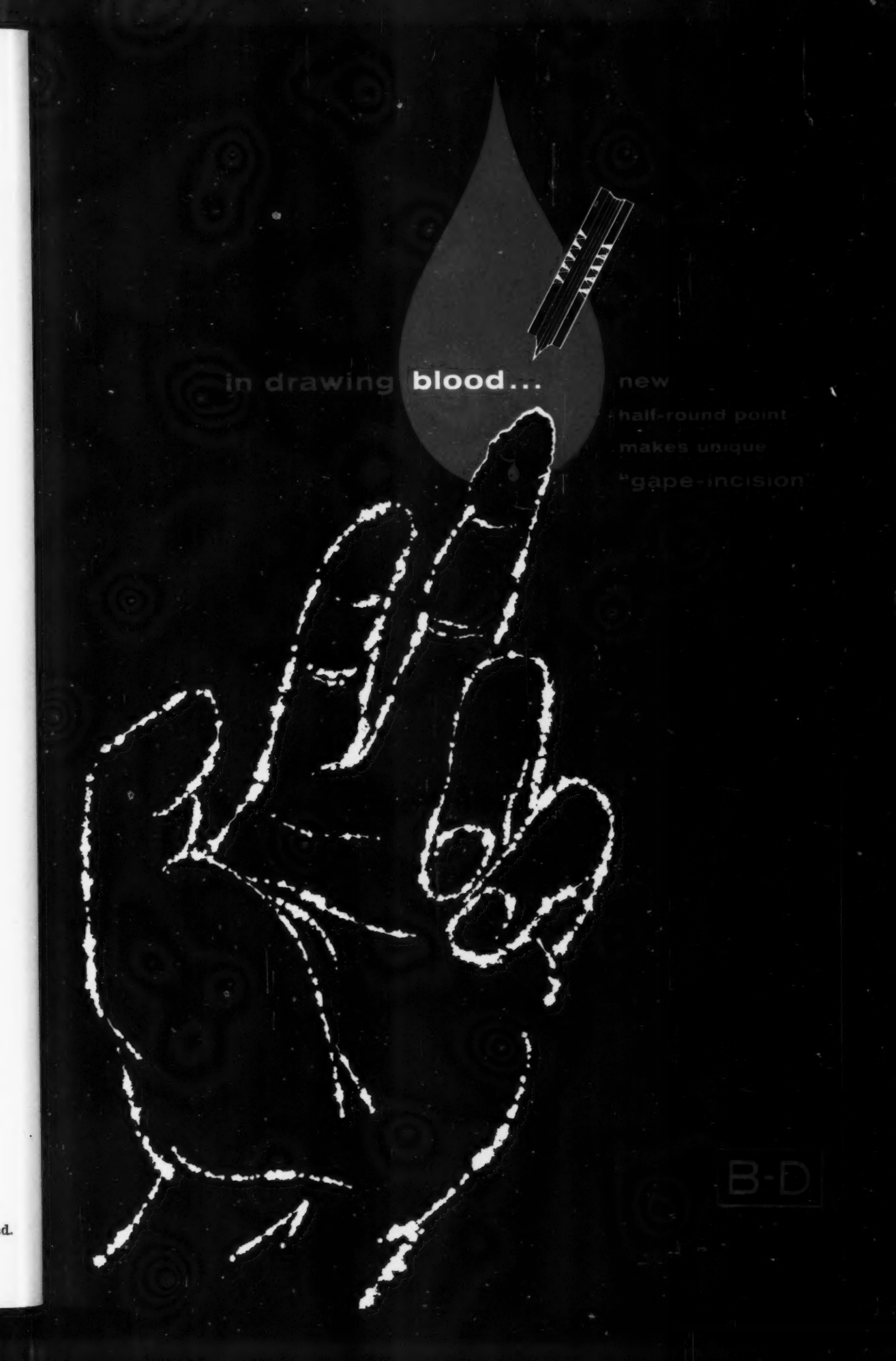
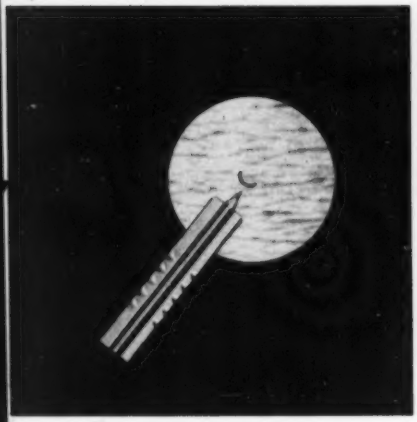
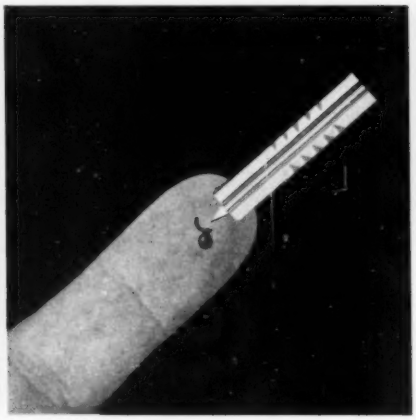

in drawing blood...
new
half-round point
makes unique
"gape-incision
B-D

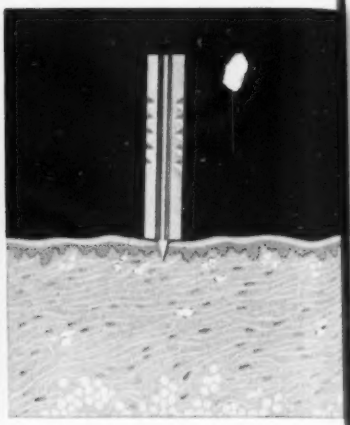
new

B-D sterile disposable blood lancet

unique "gape-incision" gives you...







adequate blood flow

The new B-D LANCET produces a half-round incision that tends to pout or "gape"—avoids premature closure—clotting delayed.

Result—an easier and immediate yield of an adequate flow of blood.

minimal dilution

The "gape-incision" of the B-D LANCET makes "milking" of the finger tip unnecessary—just gentle pressure.

Result—a minimal dilution of the blood by tissue fluids—a truer specimen of the patient's blood.

controlled penetration

Side flanges of the B-D LANCET automatically control depth of penetration. The angle and length of the point ensures that incision is in region of densest capillary supply.

Result—easy penetration—fewer tactile corpuscles traumatized—minimal pain.

- **stainless steel—very thin, yet rigid**
- **sterile—ready for immediate use**
- **hermetically sealed in pre-formed foil**
- **translucent top for positive point location**

#P433 "100" PACKAGE — Consists of 5 aluminum canisters each having 20 individually sealed LANCETS and the B-D Tallquist Hemoglobin Scale.

#433 "250" PACKAGE—Consists of 50 strips of 5 LANCETS each, individually perforated.

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Scientists, physicians and technicians who must work for long periods with a microscope will appreciate the new Leitz LABOLUX with its *fatigue-free operation, precision optics* and *unexcelled dependability*.

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Please send me your brochure on the new Leitz ***LABOLUX.***

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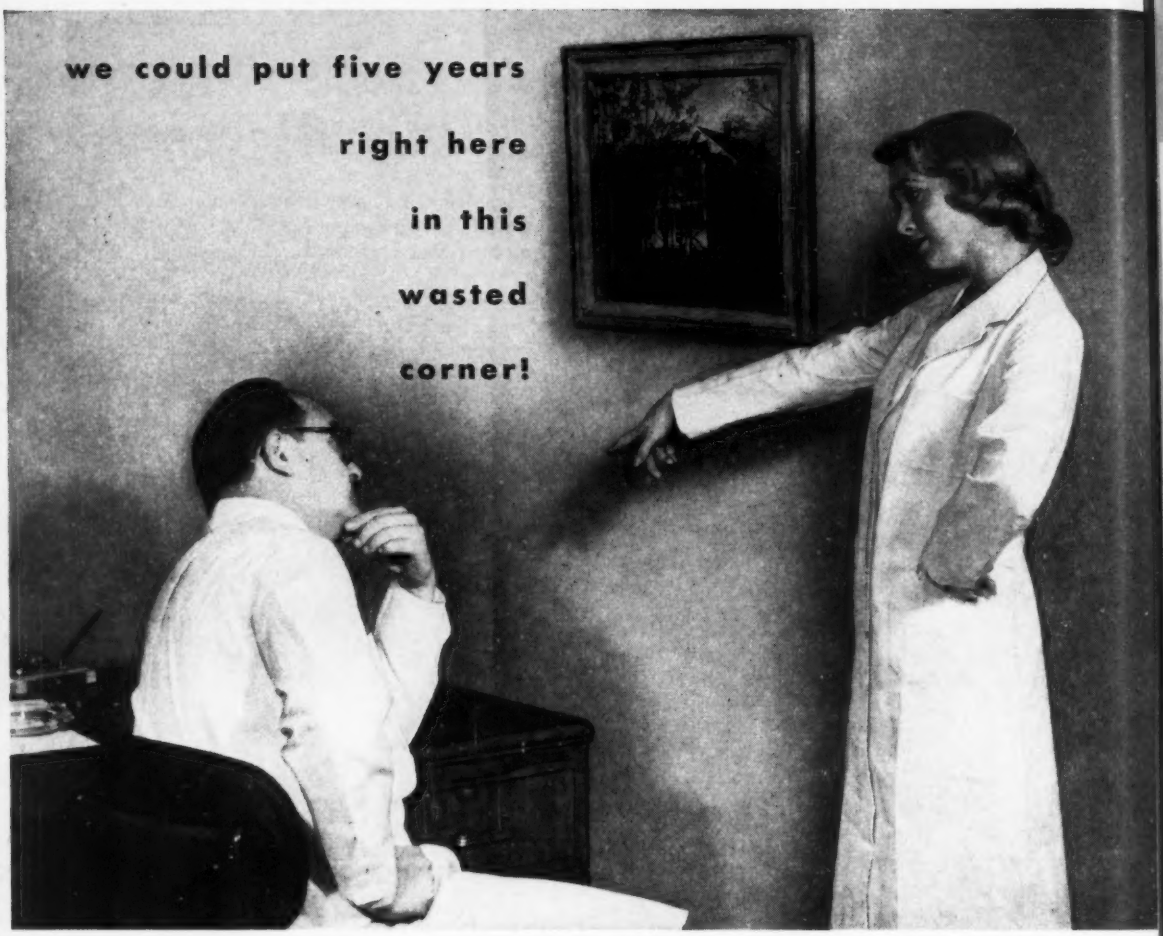
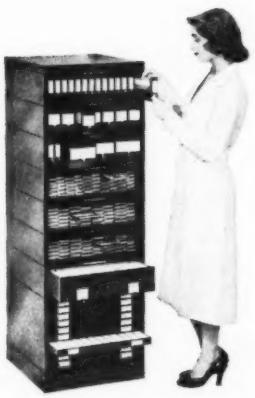


How many microslides do you average a day? Fifty, maybe?

A Lab-aid stack only 19" square and about five feet high would keep five years' output right at your fingertips.

That's because Lad-aid design gives you 45% more capacity, inch for inch, than conventional filing units. Think of it . . . a single unit section only five inches high, compact enough to fit handily on a desk top, will hold 6500 slides!



And that's not all . . . by interchanging different drawer sizes 1", 2", and 4", you can file Kodachromes, lantern slides, and index cards in the same cabinet. Other special-purpose cabinets (same basic dimensions so they all stack together) let you file fresh slides flat in spread-out trays, or file paraffin blocks in shallow drawers.

The beauty of it all is that you get all these advantages at no greater filing cost-per-slide than ordinary cabinets.

TECHNICON Lab-aid®

laboratory filing system

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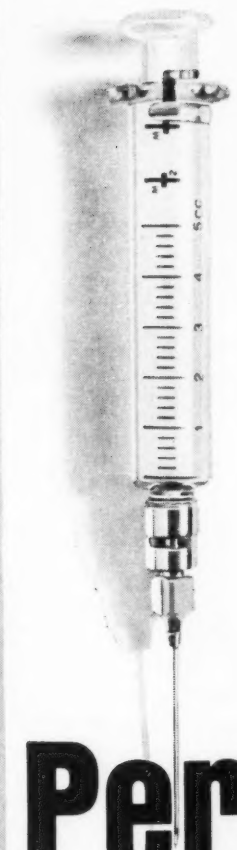

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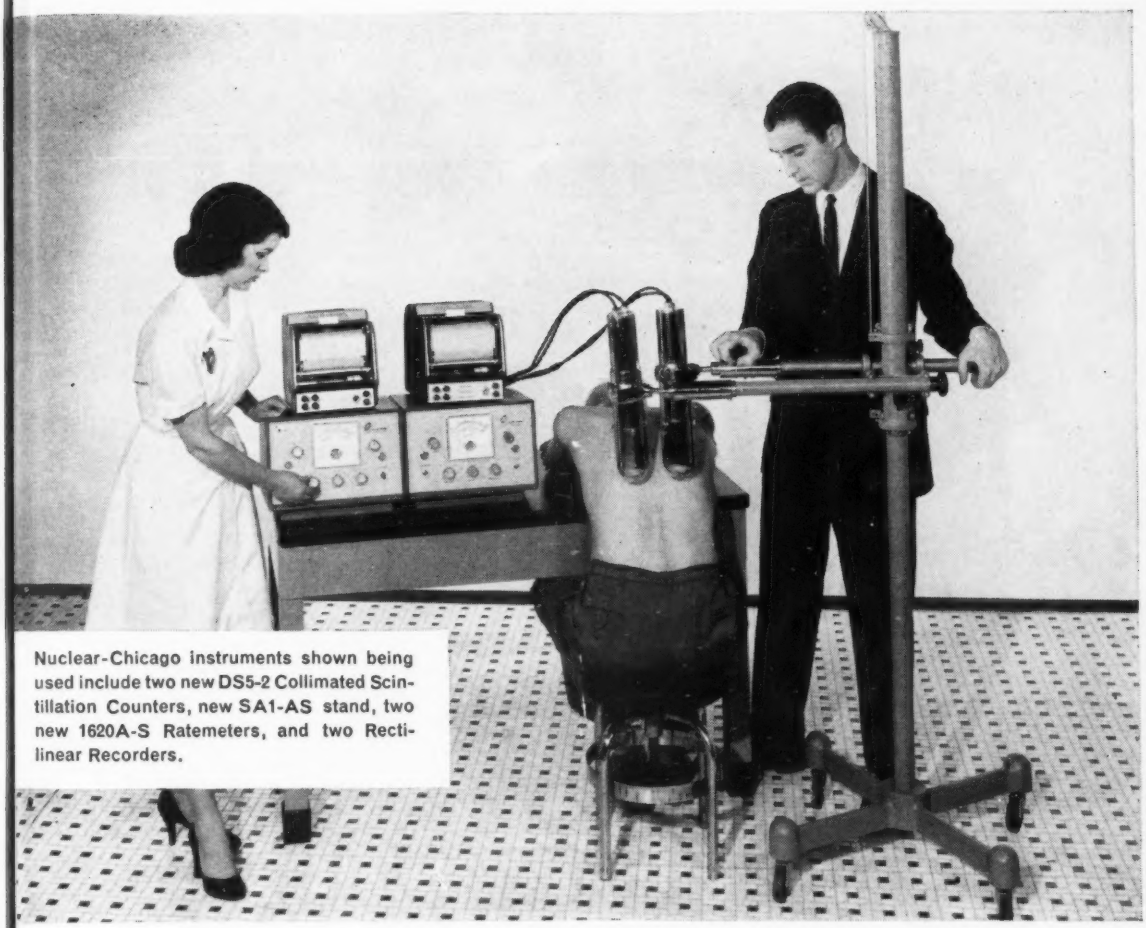

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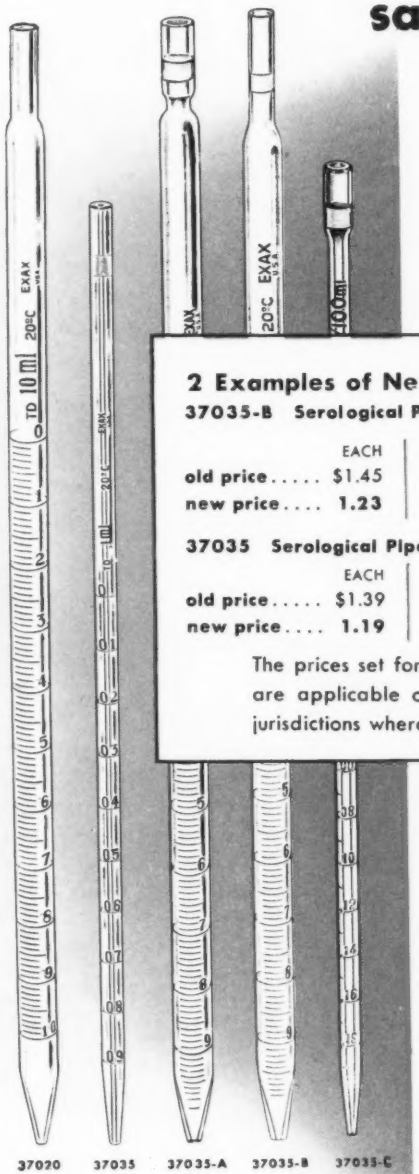

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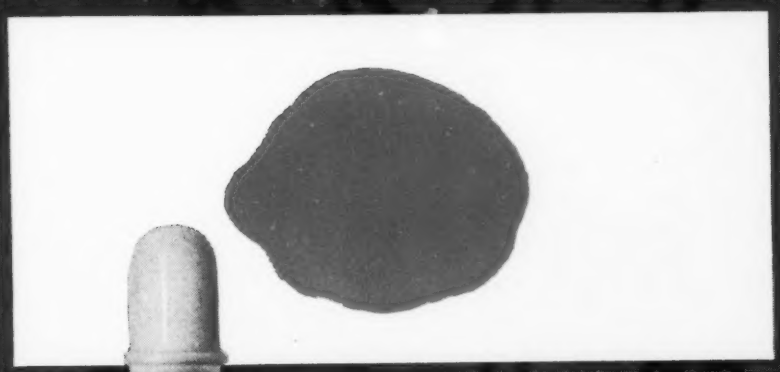

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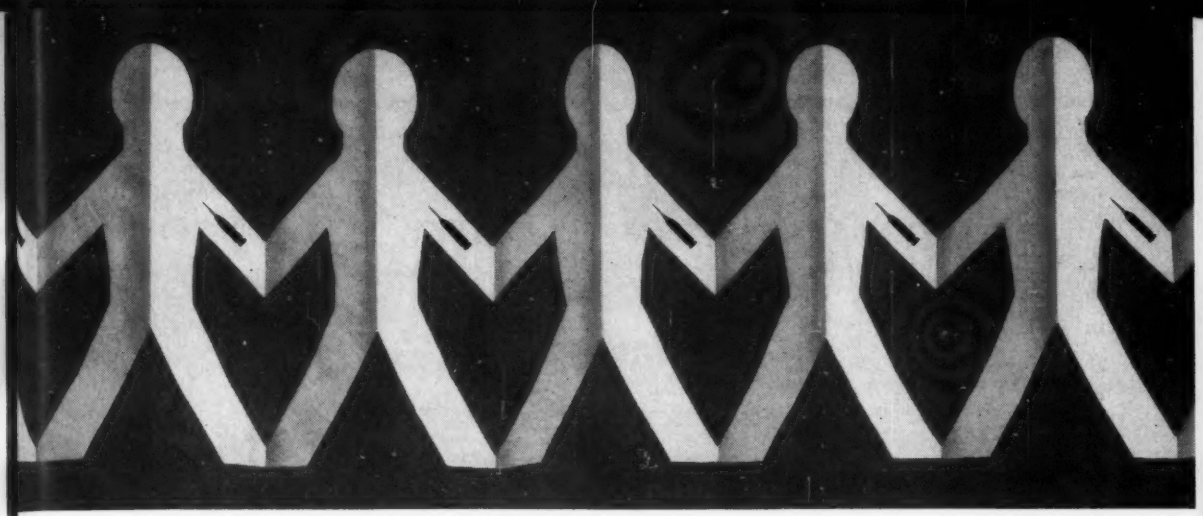

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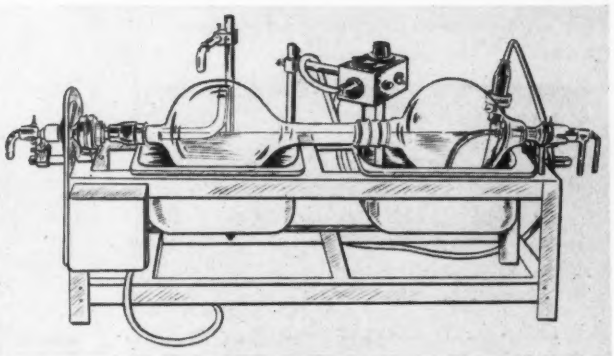

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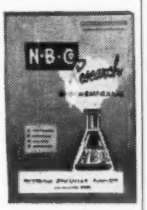

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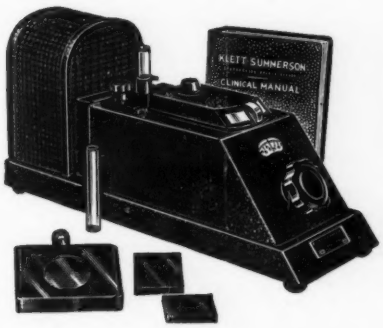

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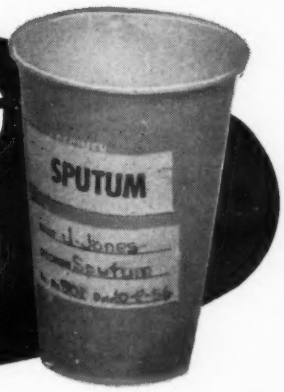

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The Official Publication of the Central Society for Clinical Research

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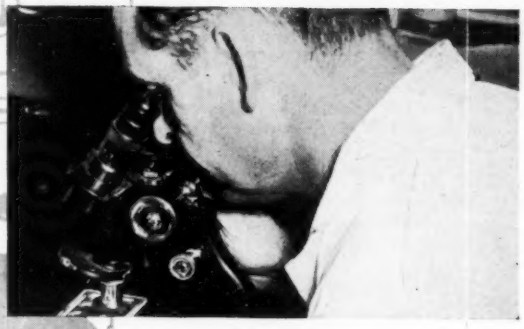

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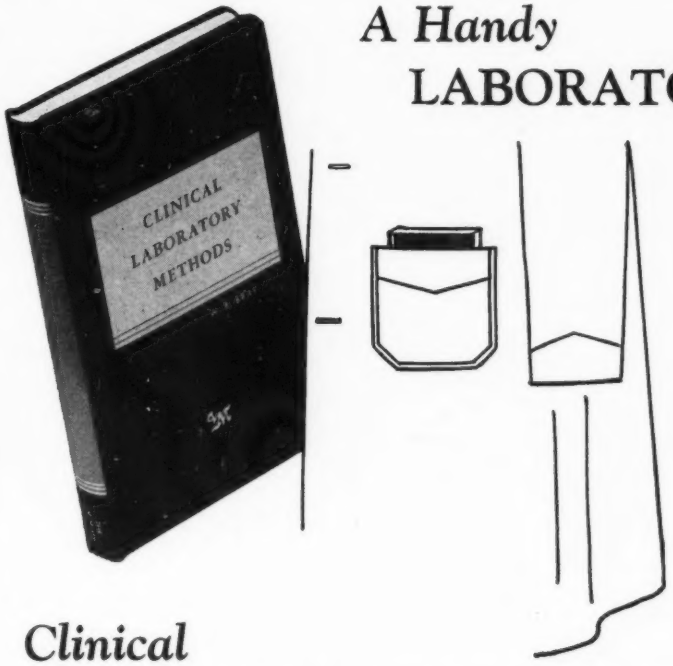

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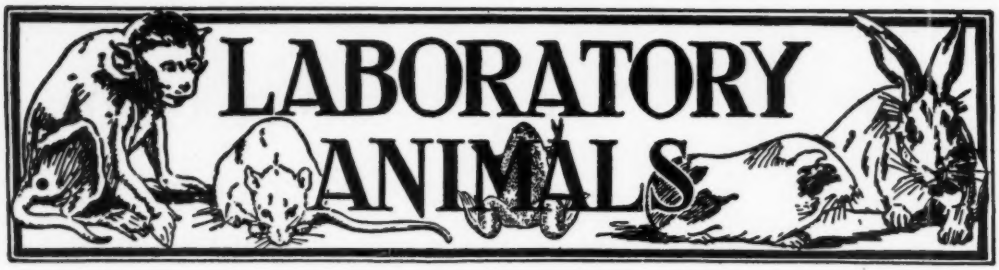

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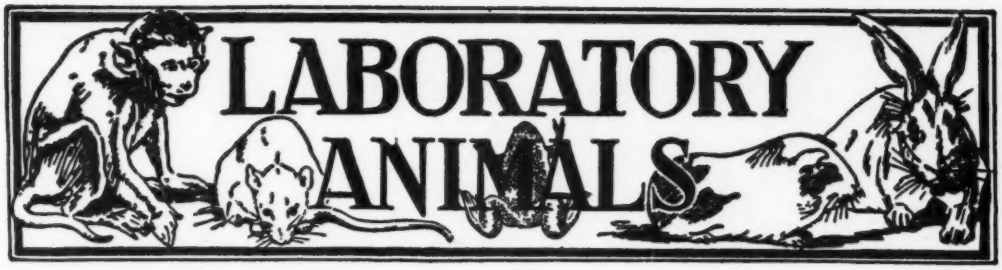

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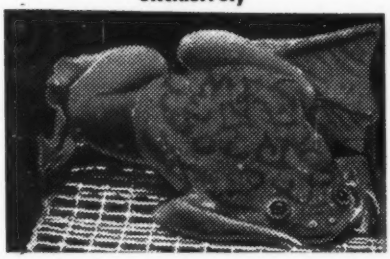

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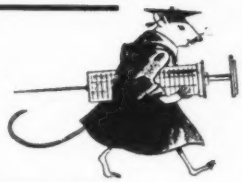

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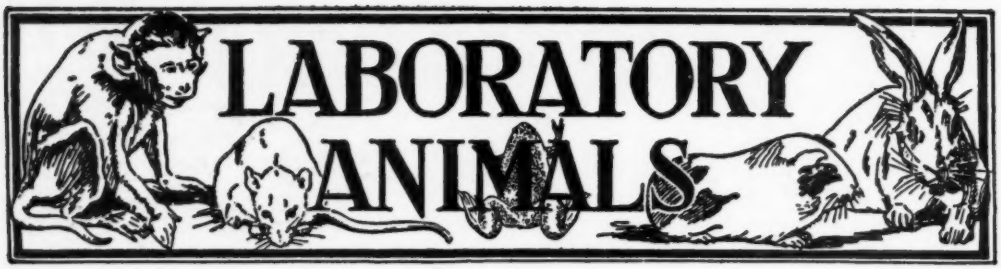

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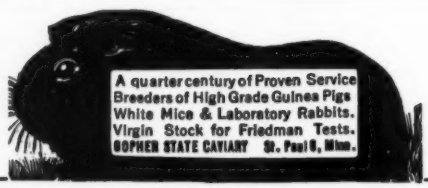
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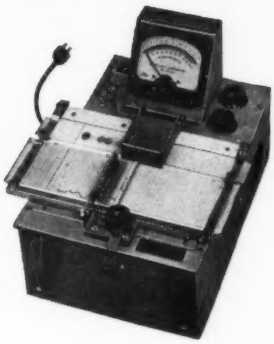

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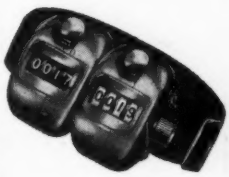

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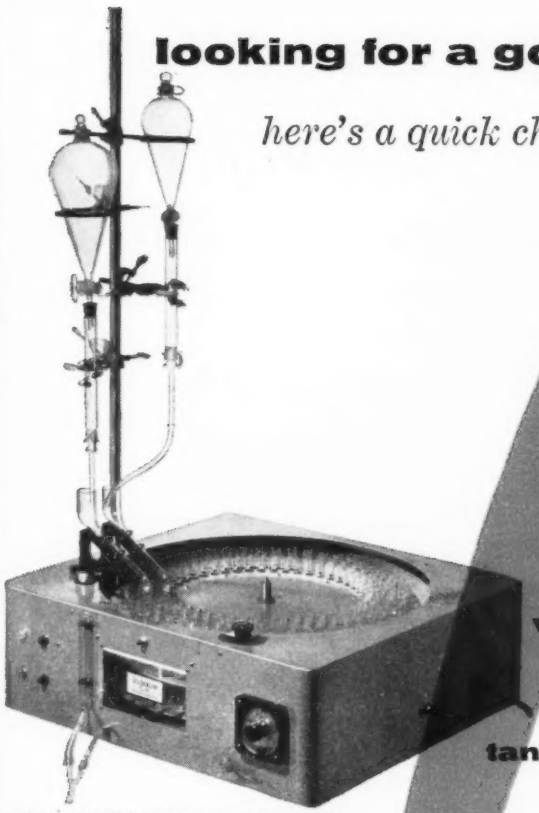

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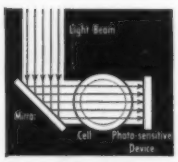

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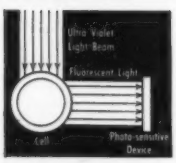

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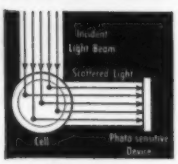

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